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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

LUTTICKEN ET AL.

Serial Number: 09/084,837 Group Art Unit: to be assigned

Filed: May 26, 1998 Examiner: to be assigned

For: RECOMBINANT BIRNAVIRUS VACCINE

CLAIM TO PRIORITY UNDER 35 USC 119

Assistant Commissioner of Patents
Washington, D.C. 20231

June 9, 1998

Sir:

The benefit of the filing date of the following prior foreign application is hereby requested for the above-identified application, and the priority provided in 35 USC 119 is hereby claimed:

European Patent Application No. 972015994 filed May 26, 1997

In support of this claim, the requisite certified copy of said original foreign application is filed herewith.

It is requested that the file of this application be marked to indicate that the Applicant has complied with the requirements of 35 USC 119 and that the Patent and Trademark Office kindly acknowledge receipt of this document.

In the event any fees are required with this paper, please charge our Deposit Account No. 02-2334.

Respectfully submitted,

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Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. / Patent application No. / Demande de brevet n°

97201599.4

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Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

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Recombinant birnavirus vaccine

The present invention is concerned with a birnavirus mutant, a vaccine comprising this mutant, a method for determining birnavirus infection in an animal, as well as with a test kit for carrying out this method.

Infectious bursal disease virus (IBDV) and Infectious pancreatic necrosis virus (IPNV) are members of the Birnaviridae family. Viruses in this family have a very similar genomic organisation and a similar replication cycle. The genomes of these viruses consist of 2 segments (A and B) of double-stranded (ds) RNA. The larger segment A encodes a polyprotein which is cleaved by autoproteolysis to form mature viral proteins VP2, VP3 and VP4 (Hudson, P.J. et al, Nucleic Acids Res., 14, 5001-50012, 1986; Dobos P., Annual review of fish diseases 5, 25-54, 1995). VP2 and VP3 are the major structural proteins of the virion. VP2 is the major host-protective immunogen of birnaviruses, and contains the antigenic regions responsible for the induction of neutralising antibodies. The VP4 protein appears to be a virus-coded protease that is involved in the processing of a precursor polyprotein of the VP2, VP3 and VP4 proteins. The larger segment A possesses also a second open reading frame (ORF), preceding and partially overlapping the polyprotein gene. This second open reading frame encodes a protein VP5 of unknown function that is present in IBDV infected cells (Mundt, E. et al., J. Gen. Virol., 76, 437-443, 1995).

The smaller segment B encodes VP1, a 90 kDa multifunctional protein with polymerase and capping enzyme activities (Spies, U. et al., Virus Res., 8, 127-140, 1987 and Spies, U. et al., J. Gen. Virol., 71, 977-981, 1990; Duncan R. et al., Virology 181, 541-552, 1991).

For IBDV, two serotypes exist, serotype 1 and 2. The 2 serotypes may be differentiated by virus neutralisation (VN) tests. Furthermore, subtypes of serotype 1 have been isolated. These so-called "variant" viruses of serotype 1 can be identified by cross-neutralisation tests (Diseases of Poultry, 9th edition, 1991, Wolfe Publishing Ltd, ISBN 0 7234 1706 7, Chapter 28, P.D. Lukert and Y.M. Saif, 648-663), a panel of monoclonal antibodies (Snyder, D.B. et al., Arch. Virol., 127, 89-101. 1992.) or RT-PCR (Jackwood, D.J., Proceedings of the International symposium on infectious bursal disease and chicken infectious anaemia, Rauischholzhausen, Germany, 155-161, 1994). Some of these subtypes of serotype 1 of IBDV have been described in literature for example: Classical, Variant-E, GLS, RS593 and DS326 strains (Van Loon, et al. Proceedings of

the International symposium on infectious bursal disease and chicken infectious anaemia, Rauischholzhausen, Germany, 179-187, 1994).

Infectious Bursal disease (IBD), also called Gumboro disease, is an acute, highly-contagious viral infection in chickens that has lymphoid tissue as its primary target with a selective tropism for cells of the bursa of Fabricius. The morbidity rate in susceptible flocks is high, with rapid weight loss and moderate mortality rates. Chicks that recover from the disease may have immune deficiencies because of the destruction of the bursa of Fabricius which is essential to the defence mechanism of the chicken. The IBD-virus causes severe immunosuppression in chickens younger than 3 weeks of age and induces bursal lesions in chicks up to 3 months old.

For many years the disease could be prevented by inducing high levels of antibodies in breeder flocks by the application of an inactivated vaccine, to chickens that had been primed with attenuated live IBDV vaccine. This has kept economic losses caused by IBD to a minimum. Maternal antibodies in chickens derived from vaccinated breeders prevents early infection with IBDV and diminishes problems associated with immunosuppression. In addition, attenuated live vaccines have also been used successfully in commercial chicken flocks after maternal antibodies had declined.

Recently, very virulent strains of IBDV have caused outbreaks of disease with high mortality in Europe. The current vaccination programs failed to protect chicks sufficiently. Vaccination failures were mainly due to the inability of live vaccines to infect the birds before challenge with virulent field virus.

Eradication of the disease by other preventative measures than vaccination has not been feasible, because the virus is widely spread and because with currently administered live attenuated or inactivated IBDV vaccines it is not possible to determine whether a specific animal is infected with an IBDV field virus or whether the animal was vaccinated with an IBDV vaccine. In order to be able to start an eradication control programme for IBDV it is highly desirable that the possibility exists to discriminate between animals vaccinated with an IBDV vaccine and those infected with a field virus so as to be able to take appropriate measures, i.e. remove infected flocks, to reduce spreading of the virulent field virus. The introduction of, for example, a serologically identifiable marker can be achieved by introducing a mutation in genes

encoding non-essential (glyco)proteins of the IBDV which still give rise to the production of antibodies in an infected host animal. A marker vaccine for Aujeszky's disease and companion diagnostic tests have proven their practical value in the control of this disease. Whereas such control programs for other viral infectious diseases in animals are under development, until the present invention a vaccine based on an IBDV vaccine strain which would fit in IBDV control programs has not been described yet. The main reason for this is that the prerequisites for the development for such an IBDV marker vaccine were not met. No permissive position or region in the genomic IBDV sequence, i.e. a position or region which can be used for the incorporation of the mutation without disrupting essential functions of IBDV, such as those necessary for infection and replication, have been identified yet. Moreover, such a non-essential region in the IBDV genome should encode a (glyco)protein which elicits a major serological response in an animal infected with wild-type IBDV, and such a region was not identified before.

The present inventors have unexpectedly found a non-essential gene within segment A of a birnavirus genome which can be mutated such that the resulting birnavirus mutant does not produce the native expression product of that gene. Moreover, it has been found that this birnavirus mutant can be used as a marker vaccine virus which allows to make a serological distinction between animals infected with wild-type birnavirus and animals immunised with a vaccine based on this birnavirus mutant.

The present invention provides a birnavirus mutant which is not able to produce a native VP5 protein as a result of a mutation in the VP5 gene of the birnavirus genome.

Preferably, the birnavirus mutant is an IBDV mutant or an IPNV mutant, the IBDV mutant being most preferred.

The inventors have found that an IBDV mutant which is not able to produce the native VP5 protein is still able to infect cells and to replicate in these cells in vitro. It is demonstrated that the IBDV mutant according to the invention is replication competent in cell culture (Example 2). The $VP5^{-}$ IBDV exhibits a delay in replication in chicken embryo cells as compared to the $VP5^{+}$ parental virus, however, final yields of the virus are similar, i.e. about $10^{7.5}$ $TCID_{50}$/ml (Example 1). Moreover, it is demonstrated that the IBDV mutant is also able to infect poultry and to replicate in the infected host animals in vivo, i.e. evidence is provided that the gene encoding the VP5 protein is a non-essential gene. Example 3 shows that the $VP5^{-}$ IBDV can be re-isolated

from organs of animals infected with the IBDV mutant and that the IBDV mutant induces a protective immune response in the infected animals.

Moreover, it has been established herein that part of the normal anti-IBDV immune response in poultry is directed to the VP5 region. This is rather surprising as the VP5 protein is considered to represent a non-structural viral protein (Mundt et al., J. Gen. Virol. 76, 437-443, 1995) and the immune response in an animal against a viral pathogen is usually elicited against the structural (glyco)proteins of the virus. These findings make the IBDV mutant and other birnavirus mutants according to the present invention a suitable vaccine candidate for a marker vaccine. Such a marker vaccine provides the possibility to determine whether animals are infected with a wild-type birnavirus, e.g. IBDV, or with a vaccine virus.

Additionally, it has been found that the VP5 protein is involved in the expression of virulence of the birnaviruses, in particular of IBDV, and that the inability of the virus mutants to produce the native VP5 protein leads to an attenuation of the virus.

With the term "which is not able to produce a native VP5 protein" is meant that the birnavirus mutant produces a polypeptide that can be distinguished by serological tests from the native VP5 protein, or does not produce a VP5 protein at all. For example, in the former case, the birnavirus mutant produces only a fragment of the native birnavirus VP5 protein which lacks one or more immunogenic epitopes.

Preferably, the birnavirus mutant according to the invention produces no VP5 protein upon infection of a host cell.

As described above, the genomic organisation of the birnaviruses is well established: the IBDV and IPNV genome comprises a large segment A and a smaller segment B. The segment A of IBDV comprises a large open reading frame (ORF) encoding a polyprotein of about 110 kDa (VP2-VP4-VP3). The gene encoding the VP5 protein is identified in the prior art, and defined herein, as the small ORF on segment A of the birnavirus genome which precedes and partially overlaps the polyprotein encoding ORF (Bayliss et al., J. Gen. Virol. 71, 1303-1312, 1990; Spies et al., J. Gen. Virol. 71, 977-981, 1990; Havarstein L.S. et al., J. Gen. Virology 71, 299-308; 1990; Dobos et al., 1995, supra; Figures 1-3 herein and SEQ ID No.'s 1-7). The mutation introduced in the VP5 gene is such that it does not prevent the expression of the polyprotein.

SEQ ID No. 1 comprises the full length cDNA nucleotide sequence of segment B of IBDV strain P2, as well as the amino acid sequence of the VP1 protein encoded by segment B (see also SEQ ID. No. 2). SEQ ID No. 3 and 5 depict the full length cDNA sequence of segment A of

IBDV strain D78 and the coding region of the VP5 protein and the polyprotein, respectively. SEQ ID 3 and 4 also show the amino acid sequence of the D78 VP5 protein. SEQ ID No. 5 and 6 show the amino acid sequence of the polyprotein VP2-VP4-VP3 of D78. SEQ ID No. 7 shows the 5'-end of segment A of strain D78, including the mutations introduced in the VP5 coding region. SEQ ID No. 8 shows the nucleotide sequence of segment B of strain D78 and the amino acid sequence of the D78 VP1 protein. The genomic organisation of both segments is also shown in Figure 1.

The ORF coding for VP5 is conserved in all hitherto published segment A sequences. The IBDV ORF encodes 145 amino acids resulting in a calculated molecular mass of 16.5 kDa. The nucleotide sequence of the ORF encoding the VP5 protein of IBDV strain D78 used herein is shown in SEQ ID No. 3 and 4. Natural variations may exist between individual IBDV isolates. These natural variations result from small differences in the genomes of these viruses. The nucleotide sequence of the segment A, including the nucleotide sequence of the VP5 gene for many IBDV isolates have been described in the prior art (Vakharia et al., Avian Diseases 36, 736-742, 1992; Bayliss et al., J. Gen. Virol. 71, 1303-1314, 1990; Hudson et al., Nuc. Acid Res. 14, 5001-5012, 1986; Schnitzler et al., J. Gen. Virol. 47, 1563-1571, 1993; Kibenge et al., J. Gen. Virol. 71, 569-577, 1990 and Virology 184, 437-440, 1991; Mundt et al., Virology 209, 10-18, 1995; Lana et al., Virus Genes 6, 247-259, 1992; Vakharia et al., Virus Res. 31, 265-273, 1994; Brown et al., Virus Res. 40, 1-15, 1996). The amino acid sequence of the VP5 protein from serotype I IBDV strains display a homology of at least 95% with the VP5 amino acid sequence shown in SEQ ID No. 3 and 4, whereas the homology between serotype II VP5 sequence and the amino acid sequence shown in SEQ ID No. 3 and 4 is at least 75%. Therefore, a preferred IBDV mutant according to the present invention is an IBDV mutant wherein the mutation is introduced in the VP5 gene having a homology of at least 75%, in particular at least 95% on the amino acid sequence level with the VP5 amino acid sequence shown herein.

Preferably an IBDV mutant according to the present invention is derived from any of the classical or variant (e.g. variant E or GLS) IBDV vaccine strains, such as those currently used in the field. Such suitable IBDV strains include the IBDV vaccine strains present in the commercially available vaccines: D78, PBG 98, LZ 228E, 89-03 (Intervet International B.V.), Bursine 2 (Fort Dodge Animal Health) and S 706 (Rhône Mérieux).

A particular preferred IBDV mutant according to the invention is derived from the D78 strain comprising a VP5 gene encoding a protein having the amino acid sequence shown in SEQ ID No. 3 and 4.

Alternatively, the parent birnavirus strain for the virus mutant according to the invention is a virulent birnavirus field strain. It is found herein that the VP5 protein is a factor associated with virulence, and that the absence of the native VP5 protein in a birnavirus results in an attenuated form of the virus.

Preferably the invention provides a birnavirus mutant which is not able to produce a native VP5 protein as a result of a mutation in the part of the VP5 gene which does not overlap with the large ORF encoding the polyprotein.

In particular, the birnavirus mutant according to the invention comprises a mutation in the 5'-end of the VP5 gene spanning nucleotides 1-30, preferably 1-20, more preferably 1-10. Most preferred is an birnavirus mutant having a mutation in nucleotides 1-3 of the VP5 gene.

A mutation is understood to be a change of the genetic information in the VP5 gene with respect to the genetic information present in this region of the genome of naturally occurring birnavirus producing native VP5 protein. The mutation is, for example, a nucleic acid substitution, deletion, insertion or inversion, or a combination thereof.

In a preferred embodiment of the present invention a birnavirus mutant is provided wherein the mutation is a substitution of one or more nucleotides. In particular, a nucleic acid substitution is introduced in the start codon, as a result of which the new codon encodes an amino acid different from methionine or represents a stop codon, preferably the nucleic acid substitution comprises at least two of the nucleotides of the start codon.

A further birnavirus mutant according to the invention comprises a substitution of one or more nucleotides in a codon(s) different from the start codon resulting in one or more stop codons, preferably in the 5'-end of the VP5 gene as defined above, if desired in addition to a substitution in the start codon as described above. Preferably, the birnavirus mutant comprises a stop codon in this region of the VP5 gene in each of the three reading frames.

Such a preferred birnavirus mutant may be an IBDV mutant having a mutation in the start codon, the fourth and the sixth codon of the VP5 gene, preferably resulting in the mutated codons shown in SEQ ID No. 7 and Figure 3.

Alternatively, a birnavirus mutant is provided wherein the mutation is a deletion. In particular, the deletion comprises less than 20, less than 10 or less than 5 nucleotides. Preferably, the deletion comprises a total number of nucleotides not dividable by three, resulting in a shift of the reading frame.

Preferably the deletion comprises one or more nucleotides of the start codon of the VP5 gene.

In an alternative embodiment of the present invention a birnavirus mutant is provided wherein the mutation comprises the insertion of a heterologous nucleic acid sequence in the birnavirus genome. A heterologous nucleic acid sequence is a nucleic acid sequence normally not present at the specific insertion site of the particular virus species.

The heterologous nucleic sequence to be incorporated into the birnavirus genome is a nucleic acid fragment which either encodes a polypeptide or is a non-coding sequence. The nucleic acid fragment can be derived from any source, e.g. viral, eukaryotic, prokaryotic or synthetic, including oligonucleotides suitable for the interruption of the expression of the VP5 gene.

A suitable oligonucleotide for the interruption of the VP5 expression may comprise three translational stop codons in each of the possible reading frames in both directions, in addition to one or more appropriate restriction enzyme cleavage sites useful for the insertion of a second heterologous nucleic acid sequence. The length and nucleotide sequence of such a non-coding heterologous nucleic acid sequence is not critical, but preferably varies between 8-50 nucleotides.

In a further embodiment of the present invention a birnavirus mutant is provided which can be used not only for the preparation of a vaccine against infection by a specific birnavirus, but also against other poultry or fish infectious diseases. For example, a vector vaccine based on such an IBDV mutant offers the possibility to immunise against other avian pathogens by the expression of antigens of these avian pathogens within infected cells of the immunised host. Such an IBDV vector according to the present invention can be obtained by inserting a heterologous nucleic acid sequence encoding a polypeptide heterologous to the IBDV in the VP5 gene as defined herein.

The heterologous nucleic acid sequence may encode an antigen of an avian pathogen such as Newcastle disease virus, Infectious bronchitis virus, Marek's disease virus, avian encephalomyelitis virus, avian reovirus, avian influenza virus, chicken anaemia virus, *Salmonella spp., E.coli, and Eimeria* spp.

Furthermore, an IBDV mutant according to the invention comprises in addition to the mutation in the VP5 gene, a mutation in the VP2 gene, wherein this gene expresses a chimeric protein comprising neutralising epitopes of more than one antigenic type of IBDV (e.g. classic, Variant-E and/or GLS). Preferably, such a mutant comprises the relevant protective VP2 epitopes of a variant GLS strain and classic strain. In particular, the mutated VP2 gene is a GLS VP2 gene comprising a nucleic acid sequence fragment encoding the B69 epitope. The construction of such a mutated VP2 genes is described in Snyder et al., Avian Diseases 38, 701-707, 1994.

Furthermore, nucleic acid sequences encoding polypeptides for pharmaceutical or diagnostic applications, in particular immuno-modulators such as lymphokines, interferons or cytokines, may be incorporated into the VP5 gene. The heterologous nucleic acid sequence may also encode a screenable marker, such as E. coli β-galactosidase or E. coli β-glucuronidase.

The construction of birnavirus mutants, in particular of IBDV mutants according to the present invention can be achieved by means of the recently established infectious cRNA system for IBDV (Mundt and Vakharia, Proc. Natl. Acad. Sci. USA 93, 11131-11136, 1996). This reverse genetics system opens the possibility to introduce mutations in the RNA genome of the IBD virus, in particular in the VP5 gene. The most important step in this reverse genetics system is to provide full length cDNA clones of the segments A and B of IBD virus. cDNA constructs comprising the segment A or B, including the nucleotides of the 5'- and 3'- ends of both these segments can be generated according to the method described by Mundt and Vakharia (1996, supra). Additionally, these constructs comprise a RNA polymerase promoter operably linked to either of the segments. The promoter can be the promoter for the T7, SP6 or T3 polymerase, the T7 promoter being preferred. Mutations can be introduced into the VP5 gene by means of methods generally known in the art for this purpose. In particular, the mutation(s) are introduced by means of site directed mutagenesis.

For example, in a first step a cDNA fragment is provided comprising at least a substantial part of the VP5 gene. In the next step suitable primer pairs are designed and hybridised with the VP5 sequence containing fragment. The 5'-primer comprises in addition to sequences complementary to the VP5 sequence, nucleotides which harbour the desired mutation, e.g. a mutation which changes the ATG start codon to an AGG (arginine) codon. Moreover, the 5'-primer is provided with an upstream nucleotide sequence representing a suitable restriction enzyme cleavage site which allows the restoring of the complete 5'-end non-coding sequence. Subsequently, the new mutated fragment is amplified using PCR and the new fragment is

introduced in the starting sequence by replacing the native nucleic acid sequence using appropriate restriction enzymes. In the next step plus-sense transcripts of the segment A and B are generated in vitro with (T7) RNA polymerease, after which the synthetic transcripts are purified using conventional RNA purification techniques. The recombinant IBDV mutant according to the invention is obtained after transfection of suitable cells (e.g. VERO cells, QM-7 cells or CEC cells) with the synthetic RNA transcripts of both segments of the IBDV genome, if desired in the presence of transfection-enhancing compositions, such as Lipofectin. Finally the recombinant IBDV is harvested from the supernatant of the transformed cells.

Methods for introducing a mutation in the birnavirus genome are described herein, but are also generally used in the art (Mundt and Vakharia, 1996, supra; Current Protocols in Molecular Biology, eds.: F. M. Ausubel et al., Wiley N.Y., 1995 edition, pages 8.5.1.-8.5.9.)

Further to the unexpected finding by the present inventors that the VP5 ORF of IBDV is a non-essential region of the IBDV genome, it has also been found that an IBDV mutant according to the present invention is able to induce a protective immune response, i.e. animals immunised with a vaccine comprising the IBDV mutant are protected against virulent challenge. Moreover, it has been found that anti-sera of animals infected with naturally occurring IBDV comprise antibodies directed to the non-structural VP5 protein and that these antisera can be distinguished from anti-sera derived from animals infected with an IBDV mutant according to the present invention. In addition, it has been found that the IBDV mutant as described above is attenuated if compared with the parent IBD virus which is able to produce the native VP5 protein.

Therefore, another aspect of this invention is a vaccine for use in the protection of animals against birnavirus infection comprising the birnavirus mutant as characterised above, together with a pharmaceutical acceptable carrier or diluent. In particular, the vaccine according to the invention is a vaccine for use in the protection of poultry against infectious bursal disease comprising the IBDV mutant described above.

The birnavirus mutant according to the present invention can be incorporated into the vaccine as live or inactivated virus.

A vaccine according to the invention can be prepared by conventional methods such as for example commonly used for the commercially available live- and inactivated IBDV vaccines. Briefly, a susceptible substrate is inoculated with an IBDV mutant according to the invention and

propagated until the virus replicated to a desired infectious titre after which IBDV containing material is harvested.

Every substrate which is able to support the replication of IBD viruses can be used in the present invention, including primary (avian) cell cultures, such as chicken embryo fibroblast cells (CEF) or chicken kidney cells (CK), mammalian cell lines such as the VERO cell line or the BGM-70 cell line, or avian cell lines such as QT-35, QM-7 or LMH. Usually, after inoculation of the cells, the virus is propagated for 3-10 days, after which the cell culture supernatant is harvested, and if desired filtered or centrifuged in order to remove cell debris.

Alternatively, the IBDV mutant is propagated in embryonated chicken eggs. In particular, the substrate on which these IBD viruses are propagated are SPF embryonated eggs. Embryonated eggs can be inoculated with, for example 0.2 ml IBDV mutant containing suspension or homogenate comprising at least 10^2 $TCID_{50}$ per egg, and subsequently incubated at 37 °C. After about 2-5 days the IBD virus product can be harvested by collecting the embryo's and/or the membranes and/or the allantoic fluid followed by appropriate homogenising of this material. The homogenate can be centrifuged thereafter for 10 min at 2500 x g followed by filtering the supernatant through a filter (100 μm).

The vaccine according to the invention containing the live virus can be prepared and marketed in the form of a suspension or in a lyophilised form and additionally contains a pharmaceutically acceptable carrier or diluent customary used for such compositions. Carriers include stabilisers, preservatives and buffers. Suitable stabilisers are, for example SPGA, carbohydrates (such as sorbitol, mannitol, starch, sucrose, dextran, glutamate or glucose), proteins (such as dried milk serum, albumin or casein) or degradation products thereof. Suitable buffers are for example alkali metal phosphates. Suitable preservatives are thimerosal, merthiolate and gentamicin. Diluents include water, aqueous buffer (such as buffered saline), alcohols and polyols (such as glycerol).

If desired, the live vaccines according to the invention may contain an adjuvant. Examples of suitable compounds and compositions with adjuvant activity are the same as mentioned below.

Although administration by injection, e.g. intramuscular, subcutaneous of the live vaccine according to the present invention is possible, the vaccine is preferably administered by the inexpensive mass application techniques commonly used for IBDV vaccination. For IBDV vaccination these techniques include drinking water and spray vaccination.

Alternative methods for the administration of the live vaccine include eye drop and beak dipping administration.

In another aspect of the present invention a vaccine is provided comprising the birnavirus mutant in an inactivated form. The major advantage of an inactivated vaccine is the extremely high levels of protective antibodies of long duration that can be achieved.

The aim of inactivation of the viruses harvested after the propagation step is to eliminate reproduction of the viruses. In general, this can be achieved by chemical or physical means. Chemical inactivation can be effected by treating the viruses with, for example, enzymes, formaldehyde, β-propiolactone, ethylene-imine or a derivative thereof. If necessary, the inactivating compound is neutralised afterwards. Material inactivated with formaldehyde can, for example, be neutralised with thiosulphate. Physical inactivation can preferably be carried out by subjecting the viruses to energy-rich radiation, such as UV light or γ-rays. If desired, after treatment the pH can be adjusted to a value of about 7.

A vaccine containing the inactivated birnavirus mutant can, for example comprise one or more of the above-mentioned pharmaceutically acceptable carriers or diluents suited for this purpose.

Preferably, an inactivated vaccine according to the invention comprises one or more compounds with adjuvant activity. Suitable compounds or compositions for this purpose include aluminium hydroxide, -phosphate or -oxide, oil-in-water or water-in-oil emulsion based on, for example a mineral oil, such as Bayol F® or Marcol 52® or a vegetable oil such as vitamin E acetate, and saponins.

The vaccine according to the invention comprises an effective dosage of the birnavirus mutant as the active component, i.e. an amount of immunising birnavirus material that will induce immunity in the vaccinated birds against challenge by a virulent virus. Immunity is defined herein as the induction of a significant higher level of protection in a population of birds after vaccination compared to an unvaccinated group.

Typically, the live vaccine according to the invention can be administered in a dose of 10^2-10^9 $TCID_{50}$ infectious $dose_{50}$ ($TCID_{50}$) per animal, preferably in a dose ranging from $10^{5.0}$-$10^{7.0}$ $TCID_{50}$, and an inactivated vaccines may contain the antigenic equivalent of 10^5-10^9 $TCID_{50}$ per animal.

Inactivated vaccines are usually administered parenterally, e.g. intramuscularly or subcutaneously.

Although, the IBDV vaccine according to the present invention may be used effectively in chickens, also other poultry such as turkeys, guinea fowl and partridges may be successfully vaccinated with the vaccine. Chickens include broilers, reproduction stock and laying stock.

The age of the animals receiving a live or inactivated vaccine according to the invention is the same as that of the animals receiving the conventional live- or inactivated IBDV vaccines. For example, broilers (free of maternally derived antibodies-MDA) may be vaccinated at one-day-old, whereas broilers with high levels of MDA are preferably vaccinated at 2-3 weeks of age. Laying stock or reproduction stock with low levels of MDA may be vaccinated at 1-10 days of age followed by booster vaccinations with inactivated vaccine on 6-8 and 16-20 weeks of age.

The invention also includes combination vaccines comprising, in addition to the IBDV or IPNV mutant according to the invention, one or more immunogens derived from other pathogens infectious to poultry or fish, respectively.

Preferably, the combination vaccine additionally comprises one or more vaccine strains of infectious bronchitis virus (IBV), Newcastle disease virus (NDV), egg drop syndrome (EDS) virus, turkey rhinotracheitis virus (TRTV) or reovirus.

In addition to a marker vaccine for birnaviruses, the availability of an appropriate diagnostic test is an essential requirement for the application of a birnavirus eradication control programme. Such a diagnostic test is provided herein and comprises a method for determining IBDV infection in poultry and IPNV infection in fish, i.e. it provides a method for distinguishing an animal in the field vaccinated with a vaccine as described above, from an animal infected with a naturally-occurring IBDV or IPNV.

Therefore, the present invention provides a method for the detection of birnavirus infection, in particular for the detection of IBDV infection in an animal comprising the step of examining a sample of the animal for the presence of VP5 antibodies or antigens. The animal is an animal from the field and is in particular an avian species, preferably a chicken. The sample coming from the animal may be any sample in which IBDV antibodies or antigens are present, e.g. a blood, serum or tissue sample, the serum sample being preferred.

A preferred method for determining birnavirus infection in an animal is a method for the detection of antibodies against the VP5 protein, comprising the steps of:

(i) incubating a sample suspected of containing anti-birnavirus antibodies, with VP5 antigen,

(ii) allowing the formation of antibody-antigen complex , and

(ii) detecting the presence of the antibody-antigen complex.

The design of this immunoassay may vary. For example, the immunoassay may be based upon competition or direct reaction. Furthermore, protocols may use solid supports or may use cellular material. The detection of the antibody-antigen complex may involve the use of labelled antibodies; the labels may be, for example, enzymes, fluorescent-, chemiluminescent-, radio-active- or dye molecules.

Suitable methods for the detection of the VP5 antibodies in the sample include the enzyme-linked immunosorbent assay (ELISA), immunofluorescent test (IFT) and Western blot analysis.

In an exemplifying ELISA, the wells of a polystyrene micro-titration plate are coated with VP5 antigen. Next, the wells of the coated plates are filled with chicken serum and serial dilutions are made. After incubation, chicken anti-VP5 protein serum antibodies are determined by detecting antibody (monoclonal or polyclonal) with the same specificity as the coated one, but which is labelled (e.g. with biotin). The labelled antibody will occupy the free antigens that have not been occupied by anti-VP5 antibodies in the chicken serum. For example, horse radish peroxidase coupled to avidin may be added and the amount of peroxidase is measured by an enzymatic reaction. If no antibodies against VP5 are present in the chicken serum sample then a maximum absorption is obtained. If the serum contains many antibodies against VP5 then a low absorption is expected. Alternatively, after the incubation with chicken serum, the amount of antibodies present in the serum that bound to the VP5 antigen may be determined directly by using an anti-chicken conjugate followed by the enzymatic reaction.

In a sandwich ELISA the wells of a polystyrene micro-titration plate can be coated with a monoclonal antibody directed against the VP5 protein. Next, the wells of these coated plates are incubated with VP5 antigen. After the antigen is captured, the wells are filled with the chicken serum and serial dilutions are made. Subsequently, the protocol as described above may be followed. This test can also be carried out by using polyclonal serum against VP5 instead of the coated monoclonal antibodies.

In another diagnostic test (Western blot analysis), the VP5 antigen (containing) material is subjected to SDS-PAGE. Next, the separated proteins are electroblotted onto nitro-cellulose

membrane. Thereafter, the membranes can be cut into lanes and the lanes are incubated with the chicken serum. The presence of VP5 antibodies in the sample can be determined by examination whether antibodies bound to the VP5 antigen, for example by using an anti-chicken conjugate followed by an enzymatic reaction. If antibodies against VP5 are present then a band at about 17 kDa is identifiable.

The VP5 antigen may be any VP5 protein (fragment) comprising material which allows the formation of the VP5 antigen-VP5 antibody complex. Preferably, the VP5 antigen comprises the expression product of a conventional recombinant host cell or virus, e.g. such as E.coli expressed VP5 (Mundt et al., J. Gen. Virol. 76, 437-443, 1995) or baculovirus expressed protein (Vakharia et al., Vaccine 12, 452-456, 1994; Vakharia et al., J. Gen Virol. 74, 1201-1206, 1993). In a further embodiment of the present invention a diagnostic test kit is provided which is suitable for performing the diagnostic test according to the invention as described above.

In particular, a diagnostic test kit is provided which comprises in addition to the components usually present, the VP5 antigen (if desired coated onto a solid phase) as the immunological reagent. Other components usually present in such a test kit include, biotin or horseradish peroxidase conjugated antibodies, enzyme substrate, washing buffer etc.

To determine birnavirus VP5 antigen in a test sample from an animal in the field, VP5-specific antibodies are used as the immunological reagent, preferably fixed to a solid phase. The test sample is added, and after an incubation time allowing formation of the antibody-antigen complex, a second labelled antibody may be added to detect the complex.

EXAMPLES

Example 1.

Construction and analysis of recombinant VP5$^-$ IBD virus

Construction of full length VP5$^-$ clone of IBDV segment A.

To construct a VP5-negative IBDV, the *Eco*RI site immediately following the 3'-end of the full length cDNA of strain D78 segment A (pUC19FLAD78; Mundt and Vakharia, Proc. Natl. Acad. Sci. USA 93, 11131-11136, 1996) was deleted. An *Eco*RI - *Kpn*I fragment containing the

T7 polymerase binding site followed by the complete segment A sequence was excised and inserted into *Eco*RI - *Kpn*I cleaved vector pUC18 after inactivation of the unique *Nde*I within the vector sequence resulting in plasmid pAD78/EK. Thereafter, the genomic region encompassing the initiation codon for VP5 was amplified in two pieces using primers A1F5' and VP5MutR, and VP5MutF and A2R, respectively (see Table 1 for sequence and location of primers). PCR fragments were cloned separately and were subsequently fused via a unique *Afl*II site which had been created by mutations within respective primers (see Fig. 2). An *Eco*RI - *Nde*I fragment containing the T7 polymerase binding site, and the 5'-part of segment A including the introduced mutations was excised and used to substitute the wild-type *Eco*RI - *Nde*I fragment in pAD78/EK to yield plasmid pAD78/VP5$^-$. Of the three mutations introduced one altered the initiation methionine codon for VP5 into an arginine codon (Fig. 2).

Table 1: Sequence of oligonucleotide primers used for generating mutant constructs.

[a]Nucleotide sequence	Orientation	Designation	Nucleotide no.
AGAGAATTC*TAATACGACTCACTATA*GGA TACGATCGGTCTGAC	+	A1F5'	1-18
TGGGCCTGTCACTGCTGTCACATGT	-	A2R	716 - 740
CATTGCTCTGCAGTGTGTAGTGAGC	-	A3R	338 - 362
CTACAACGCTATCC**TT**AAGGGTTAGTA GAG	+	VP5MutF	80 - 109
CTCTACTAACCC**TT**AAGGATAGCGTTGT AG	-	VP5MutR	80 - 109

a) Underlined nucleotides denote virus specific nucleotides. T7 promotor sequences are marked in italics. Mutated nucleotides are bold and orientation of the primer is shown for sense (+) and antisense (-). Primer positions are given according to the published sequence of serotype I strain P2 (Mundt et al., Virology 209, 209-218, 1995).

Virus recovery from cRNA. For *in vitro* transcription of RNA plasmids pAD78/EK, pAD78/VP5$^-$ and pBP2 (Fig. 2) were linearized by cleavage with *Bsr*GI and *Pst*I, respectively. Treatment of linearized DNA, transcription and purification of RNA, and transfection were carried out as described by Mundt and Vakharia (1996, supra) with the exception that secondary

CEC were used for the transfection experiments. Three days after transfection a CPE was visible in CEC. Cells were freeze/thawed, centrifuged at 700 x g to eliminate cellular debris, and the resulting supernatants were filtrated through 0.45 μm filters and stored at -20°C. For the transfection experiments full length cDNA clones of segment A of strain D78 capable of expressing (pAD78/EK) or unable to express VP5 (pAD78/VP5⁻) were transcribed into synthetic RNA and cotransfected with segment B full length cRNA into CEC. Resulting virus progeny IBDV/EK and IBDV/VP5⁻ was further characterised.

Analysis of transfection progeny by immunofluorescence and Radioimmunoprecipitation assay (RIPA). VP5 was expressed in E.coli as described in Mundt et al. (J. Gen. Virol. 76, 437-443, 1995). Rabbit monospecific polyclonal anti serum and mouse monoclonal antibodies against VP5 were prepared according to standard protocols. Vero cells infected with IBDV/VP5⁻, IBDV/EK, and non-infected cells , respectively, were incubated with rabbit anti-IBDV serum, rabbit anti-VP5 serum and with anti-VP5 mAb DIE 7, and stained with fluoresceine-conjugated secondary antibodies. Both antisera and the monoclonal antibody recognised IBDV antigens in the cytoplasm of IBDV/EK infected cells. In contrast, whereas the anti-IBDV serum readily detected viral antigens in IBDV/VP5⁻ infected cells, neither the monospecific anti VP5-serum nor the monoclonal anti-VP5 antibody exhibited specific reactivity. None of these immunological reagents reacted with non-infected controls.

To analyse viral proteins expressed during replication lysates of radioactively labelled CEC infected with IBDV/VP5⁻ (Fig 4, lanes 1-3) and IBDV/EK (Fig. 4, lanes 4-6) were immunoprecipitated with rabbit anti-IBDV serum, rabbit anti-VP5 serum and mAb DIE 7. Non-infected CEC were used as control (Fig. 4, lanes 7-9). IBDV/EK (lane 4) as well as IBDV/VP5⁻ (lane 1) infected CEC showed viral proteins VP2, VP3, and VP4 after precipitation with rabbit anti-IBDV serum. The rabbit anti-VP5 serum (lane 5) and mAb DIE 7 (lane 6) precipitated VP5 with a molecular mass of 21 kDa only from IBDV/EK infected cells. No specific reactivity was detectable in IBDV/VP5⁻ infected CEC after precipitation with rabbit-anti VP5 (lane 2) as well as the VP5 specific mAb DIE 7 (lane 3). Non-infected CEC showed no specific reactivity (lanes 7-9).

Replication of IBDV/VP5⁻ in CEC. To assay replication of IBDV/VP5⁻ in more detail one step growth was analysed (Fig. 5). Confluent secondary CEC were infected with IBDV/EK and IBDV/VP5⁻ with $10^{7.2}$ $TCID_{50}$, respectively. Immediately after overlaying the infected cells with 5 ml growth medium, supernatant from one infected CEC tissue plate of each virus was removed and stored at -20°C (0 h p.i.). Remaining tissue culture plates were further incubated and 4h, 8h, 16h, 24h, and 48h p.i. supernatants were removed and stored at -20°C. Supernatants were centrifuged and titrated according to standard methods. The $TCID_{50}$ at the different time points after infection showed that the VP5 expressing virus (IBDV/EK) replicated faster than the virus mutant lacking VP5 (IBDV/VP5⁻). 16 h after infection IBDV/EK showed a 100-fold higher than IBDV/VP5⁻ (Fig. 5). However, at 48 h p.i. IBDV/VP5⁻ reached a titre of $10^{7.2}$ $TCID_{50}$/ml which was similar to IBDV/EK ($10^{7.45}$/ml)

Preparation of recombinant IBDV VP5⁻-2. Plasmid pAD78/VP5⁻-2 was prepared by techniques similar to those described above. The nucleotide sequence of part of the mutated VP5 gene is shown in SEQ ID No. 7 and Figure 3. A restriction enzyme fragment harbouring the mutations was used to substitute the wild-type *Eco*RI - *Nde*I fragment in pAD78/EK. An outline of the protocol for the preparation of the recombinant plasmid is shown in Figure 3. The organisation of pBD78 is also depicted in Figure 3. The recombinant virus was prepared as described above, except for the fact that segment B of strain D78 (SEQ ID No. 8) was used and QM-7 cells were used for the transfection experiment.

Example 2

Identification of VP5 protein in different IBDV strains

Different strains of IBDV were investigated for the expression of the VP5-gene. This was done by making use of the immuno-fluorescence technique (IFT). Chicken embryo fibroblasts grown in microtiterplates were infected with different IBDV strains. Three to 5 days after incubation at 37°C cells were fixed with 70% ethanol, then treated with polyclonal rabbit anti IBDV serum (R1928), polyclonal rabbit anti VP5 serum (RαVP5) or monoclonal antibody directed against VP5 (DIE7), respectively. Binding of the poly- or monoclonal antibodies to the

different IBDV strains was visualised by making use of a fluorescence labelled conjugate (goat-anti-rabbit or goat-anti-mouse). The results are shown in Table 2:

Table 2: Identification of different sero- and subtypes of IBDV strains. Determination of the presence of VP5 proteins.

IBDV-serotype	IBDV-subtype	IBDV-strain	R1928	RαVP5	DIE7
I	Classical	D78	+	+	+
I	Classical	228TC	+	+	+
I	Classical	PBG98	+	+	+
I	Classical	Ram0404	+	+	+
I	Classical	IBDV/EK	+	+	+
I	Classical	IBDV/VP5$^-$	+	-	-
I	GLS	GLS	+	+	+
I	Variant-E	8903	+	+	+
II	TY89	TY89	+	+	+

From these data it can be concluded that the different strains of IBDV belonging to different sero- and subtypes do express the VP5-gene. Furthermore, the recombinant VP5$^-$ IBDV vaccine strain can be differentiated from field and vaccine viruses, thereby enabling the recombinant VP5$^-$ virus to be used as a marker vaccine.

Example 3

In vivo testing of the recombinant VP5$^+$ and VP5$^-$ IBDV vaccines in comparison with a commercial available live IBDV vaccine.

Preparation of IBDV vaccine. Primary chicken embryo fibroblast (CEF) cells were prepared at a final concentration of $2x10^6$/ml. The cells were cultured in Eagles minimum essential medium containing 5% fetal calf serum. To 25 ml of this cell suspension 0.1 ml

IBDV/EK or IBDV/VP5^{-} virus (having an infectious titre of about 3.0 log10 $TCID_{50}$/ml) was added. After incubation for 5 days in a high-humidity incubator at 37°C, the total suspension was used in the animal experiment without further purification. The infectious titre of the supernantant was $10^{7.1}$ TCID50/ml.

Animal experiment. In this study the potency of different vaccines (VP5 positive strain IBDV/EK and a VP5 negative strain IBDV/VP5^{-}, and the commercial available IBDV vaccine Nobilis strain D78, Intervet International B.V., NL) was investigated. SPF chicks of 3 weeks old were treated as indicated in the treatment schedule.

Treatment Schedule:

Days after vaccination	Groups			
	1	2	3	4
00	IBDV/EK	IBDV/VP5^{-}	D78	-
03	x	x1	x	x
07	x,bl	x1,bl	x,b	x,bl
14	x,bl	x,bl	x,bl	x,bl
20	x,bl	x,bl	x,bl	x,bl
21	ch	ch	ch	ch
24	x	x	x	x
31	+	+	+	+

VP5^{+} Bursal disease vaccination with VP5 positive vaccine clone, eye-drop route, dose $10^{4.6}$ TCID50/animal, 0.1 ml/animal.

VP5^{-} Bursal disease vaccination with VP5 negative vaccine clone, eye-drop route, dose $10^{5.9}$ TCID50/animal, 0.1 ml/animal.

D78 Bursal disease vaccination with IBDV VACCINE NOBILIS STRAIN D78, eye-drop route, one field dose.

ch Challenge with Bursal disease virus, Farragher strain F52/70, eye-drop route, dose $10^{2.0}$ CID50/animal, 0.1 ml/animal.

bl Serological examination; VN-test and/or Western blotting.

x Histological examination (H.E. staining) and MCA-8 ELISA on bursae.

x1 Histological examination (H.E. staining) and MCA-8 ELISA on bursae and reisolation of virus from bursa of Fabricius.

\+ Clinical examination and after 10 days histological examination of the bursa.

Detection of virus in the bursa of Fabricius.

Three, 7, 14 and 20 days after eye-drop vaccination, animals were sacrificed and blood and bursae obtained. The presence of virus in the bursa was determined with an enzyme-linked immunosorbent assay (ELISA) making use of the monoclonal antibody 8 (MAB-8). MAB-8 is directed specifically against IBDV. Data are depicted in Table 3.

Furthermore, 3 and 7 days after vaccination, bursae from animals of group 2 were investigated for the presence of the recombinant $VP5^-$ virus. For that purpose bursae were homogenised and cultured on chicken embryo fibroblasts. The presence of the $VP5^-$ virus was determined by IFT using polyclonal rabbit sera against IBDV or VP5 or monoclonal antibodies against VP5. From 13 out of 15 bursae (87%) investigated, $VP5^-$ virus could be reisolated and identified (positive for R1928 and negative for RαVP5 and DIE7). This indicates that the virus upon animal passage is still $VP5^-$, indicating that the virus is stable and does not revert to $VP5^+$. Furthermore, by using the different poly- and monoclonal antibodies $VP5^-$ vaccine virus can be discriminated from all other vaccine and/or field IBDV viruses. Therefore, the $VP5^-$ vaccine may be used as a marker vaccine.

Three days after challenge no virus could be detected in groups 1, 2 and 3 with the MCA-8 ELISA. In contrast, all animals of group 4 (non-vaccinated control group) contained challenge virus in the bursa of Fabricius, 3 days after challenge. The results show that animals vaccinated with recombinant $VP5^+$ (group 1), recombinant $VP5^-$ (group 2) and IBDV vaccine Nobilis D78 (group 3) were protected against severe challenge.

Table 3: Individual data for detection of virus in the bursa of Fabricius with the MCA-8 ELISA at different days after vaccination or challenge.

	Days after vaccination→				Days after challenge	
	3	7	14	20	3	
Group↓	Virus detection by ELISA↓					Protection↓
1 VP5$^+$	2/8*	1/7	0/2	0/3	0/5	100%
2 VP5$^-$	0/8	0/7	0/2	0/3	0/5	100%
3 D78	1/8	6/7	0/2	0/3	0/5	100%
4 -	0/8	0/7	0/2	0/3	5/5	0%

*Number of positive bursae per total number tested.

Detection of lesions in the bursa of Fabricius.

The microscopic average lesion score induced by the different IBDV (recombinant) vaccines or the challenge virus are depicted in Table 4.

Before challenge, animals vaccinated with the recombinant VP5$^+$ IBDV vaccine (group 1) or vaccinated with IBDV vaccine Nobilis D78 (group 3) showed mild to moderate lesions in the bursa. Three days after challenge only chronic lesions were observed in the bursa of Fabricius, indicating that the animals of groups 1 and 3 were protected against challenge. Furthermore, 10 days after challenge only very mild lesions (0-20% lymphocytic depletion) were observed in the bursa of the animals vaccinated with VP5$^+$ recombinant IBDV vaccine or with Nobilis vaccine D78. In contrast animals not vaccinated and challenged showed severe lesions 10 days after challenge. In other words all animals (100%) of groups 1 and 3, vaccinated with the VP5$^+$ recombinant IBDV vaccine or with Nobilis vaccine D78 were protected against severe challenge.

Three, 7, 14 and 20 days after vaccination and 3 and 10 days after challenge with the recombinant VP5$^-$ IBDV vaccine, animals of group 2 showed no to hardly any lesions (0-20% lymphocytic depletion) in the bursa. All animals of group 2, vaccinated with the VP5$^-$ recombinant IBDV vaccine, were protected against severe challenge. When animals vaccinated with the recombinant VP5$^-$ IBDV vaccine are compared to animals of groups 1 or 3 (vaccinated

with a recombinant $VP5^+$ or commercial available vaccine) the recombinant $VP5^-$ vaccine induces less lesions and therefore, is safer, milder than the vaccines tested in this experiment.

Three days post-challenge, all non-vaccinated animals of group 4 showed severe acute lesions in the bursa (total lymphocyte depletion, score 5.0). Ten days after challenge, all animals (17 out of 17 animals) showed total lymphocytic depletion, indicating that these animals were not protected against severe challenge. Animals that died after challenge, all showed severe lesions in the bursa of Fabricius. It was concluded that control group 4 was not protected against severe challenge indicating that the test conditions were optimal.

Table 4: Average bursal lesion score at different days after vaccination or challenge. The average lesion score is calculated as follows: all lesion scores from the animals per group on a certain day are added. This number is then divided by the total number of animals investigated in that group on that day. Individual scores range from 1 to 5. Score 0 = no lymphocytic depletion, score 1 = 0 - 20%; score 2 = 20 - 40%; score 3 = 40 - 60%; score 4 = 60 - 80% and score 5 = 80 - 100 % lymphocytic depletion (total lymphocytic depletion).

	Days after vaccination→				Days after challenge→		
	3	7	14	20	3	10	
Group↓	Bursal lesions score↓						Protection↓
1 $VP5^+$	0.8	2.9	1.0	1.0	1.0^c	0.6	100%
2 $VP5^-$	0.0	0.0	0.5	0.0	0.0^c	0.1	100%
3 D78	0.1	2.4	3.5	2.0	2.8^c	1.1	100%
4 -	0.0	0.0	0.0	0.0	5.0^a	5.0	0%

[a] Acute lesions [c] Chronic lesions

Serological response.

The serological response of the animals was determined by measuring the ability of blood serum to neutralise a classical infectious bursal disease virus strain in a virus neutralising (VN) test. Serum was investigated 3, 7, 14 and 20 days after vaccination. The average neutralising titres are shown in Table 5.

The results show that recombinant IBDV vaccine $VP5^+$ applied to chickens of group 1 induced a good and high serological response 20 days after vaccination which is comparable to the serological response of the chickens vaccinated with the commercial IBDV vaccine Nobilis strain D78 (group 3). The recombinant IBDV vaccine $VP5^-$ applied to chickens of group 2 induced also a good serological response. A titre of 9.4 log2 was observed 20 days after vaccination. The serological response induced by the recombinant $VP5^-$ IBDV vaccine was delayed when compared to the serological response induced by the recombinant IBDV $VP5^+$ vaccine or the commercial IBDV vaccine Nobilis strain D78.

The non-vaccinated group 4 showed no serological response to IBDV.

Table 5: Average IBDV-VN-titres for groups 1 to 4 at different days after vaccination, expressed as log2 of the dilution.

Group	Days after vaccination			
	3	7	14	20
1 $VP5^+$	$\leq 1.0 \pm 0.0$	7.1 ± 1.7	10.2 ± 1.4	11.9 ± 1.8
2 $VP5^-$	$\leq 1.0 \pm 0.0$	2.1 ± 1.7	6.3 ± 2.9	9.4 ± 1.4
3 D78	$\leq 1.0 \pm 0.0$	5.2 ± 2.8	10.3 ± 1.3	11.6 ± 1.5
4 -	$\leq 1.0 \pm 0.0$	$\leq 1.0 \pm 0.0$	$\leq 1.0 \pm 0.0$	$\leq 1.0 \pm 0.0$

Serological differentiation between antisera.

The serological response against VP5 was investigated by making use of western blot analysis. For this purpose the VP5 protein was expressed in the E. coli or baculo expression system. The expressed proteins were separated by SDS PAGE. Next the proteins were electroblotted onto a nitro-cellulose membrane. Thereafter, the membrane was cut into lanes and the lanes were incubated with rabbit anti-VP5 serum, chicken serum directed against $VP5^+$ recombinant vaccine, chicken serum directed against $VP5^-$ recombinant vaccine or negative serum from SPF chickens. Data are summarised in Table 6. As can be seen from Table 6, the $VP5^-$ serum does not induce a serological response against VP5. In contrast the rabbit anti-VP5 serum and chicken serum directed against $VP5^+$ recombinant vaccine do recognise the VP5-

protein and thus induces a serological response against VP5. This indicates that chicken serum may be used to investigate if animals are exposed to a virus that expresses the VP5 protein (e.g. field virus) or to the $VP5^-$ recombinant vaccine.

Table 6: Western blot analysis. Serum from animals vaccinated with $VP5^+$ or VP5- recombinant vaccine as well as SPF chicken serum and anti VP5-rabbit serum were investigated for their reaction with the VP5-protein.

Identification of serum sample	Immuno-blot
$VP5^+$ vaccinated animal, serum sample 20d after vaccination	positive
$VP5^-$ vaccinated animal, serum sample 20d after vaccination	negative
Non-vaccinated control, serum sample at 20d	negative
Rabbit anti VP5 serum	positive

Mortality and clinical signs.

None of the animals vaccinated with $VP5^+$ IBDV vaccine (group 1), vaccinated with recombinant $VP5^-$ IBDV vaccine (group 2) or vaccinated with the commercial IBDV vaccine Nobilis strain D78 (group 3), died or showed clinical signs of infectious bursal disease after challenge, indicating that the animals were protected against severe challenge. All animals in the non-vaccinated control group were not protected against severe challenge.

LEGENDS TO THE FIGURES

Figure 1 Genomic organization of segment A and segment B of IBDV. The numbers indicate the nucleotide positions of the start, end and coding region on the segments.

Figure 2 Construction of genomic cDNA clones for the preparation of IBDV/VP5$^-$. Plasmid pAD78/EK contains the complete D78 segment A cDNA encoding the polyprotein (VP2-VP4-VP3) and VP5. Plasmid pBP2 contains the complete strain P2 segment B encoding VP1. Mutations were introduced in plasmid pAD78/VP5$^-$ altering the methionine start codon for VP5 into arginine and creating an artificial Afl II cleavage site. Recombinant plasmids were linearized with the underlined restriction enzymes, followed by T7 polymerase transcription.

Figure 3 Construction of genomic cDNA clones for the preparation of IBDV/VP5$^-$-2 . Plasmid pAD78/EK contains the complete D78 segment A cDNA encoding the polyprotein (VP2-VP4-VP3) and VP5. Plasmid pBD78 contains the complete strain D78 segment B encoding VP1. Mutations were introduced in plasmid pAD78/VP5$^-$ altering the methionine start codon for VP5 into glutamic acid and creating an artificial BstBI cleavage site. Further mutations were introduced in the arginine and glutamine codon. Recombinant plasmids were linearized with the underlined restriction enzymes, followed by T7 polymerase transcription.

Figure 4 Radioimmunoprecipitation of proteins from CEC infected cells with recombinant IBDV. CEC infected cells with IBDV/VP5$^-$ (lanes 1-3), IBDV/EK (lanes 4-6) and uninfected controls were immunoprecipitated with rabbit anti-IBDV serum (lanes 1, 4, 7), rabbit anti-VP5 serum (lanes 2, 5, 8) and mAb DIE 7 (lanes 3, 6, 9). Position of molecular mass markers (M) is indicated. Location of the viral proteins VP2, VP3, VP4 and VP5 are marked.

Figure 5 Replication kinetics of IBDV/EK and IBDV/VP5$^-$. Infectious titers of supernatants (vertical axis) are determined at the times indicated.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Azko Nobel N.V.
(B) STREET: Velperweg 76
(C) CITY: Arnhem
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(ii) TITLE OF INVENTION: Recombinant birnavirus vaccine

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2827 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:112..2745

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGATACGATG GGTCTGACCC TCTGGGAGTC ACGAATTAAC GTGGCTACTA GGGGCGATAC 60

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CCGCCGCTGG CCGCCACGTT AGTGGCTCCT CTTCTTGATG ATTCTGCCAC C ATG AGT      117
                                                         Met Ser
                                                           1

GAC ATT TTC AAC AGT CCA CAG GCG CGA AGC ACG ATC TCA GCA GCG TTC      165
Asp Ile Phe Asn Ser Pro Gln Ala Arg Ser Thr Ile Ser Ala Ala Phe
            5                  10                  15

GGC ATA AAG CCT ACT GCT GGA CAA GAC GTG GAA GAA CTC TTG ATC CCT      213
Gly Ile Lys Pro Thr Ala Gly Gln Asp Val Glu Glu Leu Leu Ile Pro
     20                  25                  30

AAA GTT TGG GTG CCA CCT GAG GAT CCG CTT GCC AGC CCT AGT CGA CTG      261
Lys Val Trp Val Pro Pro Glu Asp Pro Leu Ala Ser Pro Ser Arg Leu
 35                  40                  45                  50

GCA AAG TTC CTC AGA GAG AAC GGC TAC AAA GTT TTG CAG CCA CGG TCT      309
Ala Lys Phe Leu Arg Glu Asn Gly Tyr Lys Val Leu Gln Pro Arg Ser
                 55                  60                  65

CTG CCC GAG AAT GAG GAG TAT GAG ACC GAC CAA ATA CTC CCA GAC TTA      357
Leu Pro Glu Asn Glu Glu Tyr Glu Thr Asp Gln Ile Leu Pro Asp Leu
             70                  75                  80

GCA TGG ATG CGA CAG ATA GAA GGG GCT GTT TTA AAA CCC ACT CTA TCT      405
Ala Trp Met Arg Gln Ile Glu Gly Ala Val Leu Lys Pro Thr Leu Ser
         85                  90                  95

CTC CCT ATT GGA GAT CAG GAG TAC TTC CCA AAG TAC TAC CCA ACA CAT      453
Leu Pro Ile Gly Asp Gln Glu Tyr Phe Pro Lys Tyr Tyr Pro Thr His
    100                 105                 110

CGC CCT AGC AAG GAG AAG CCC AAT GCG TAC CCG CCA GAC ATC GCA CTA      501
Arg Pro Ser Lys Glu Lys Pro Asn Ala Tyr Pro Pro Asp Ile Ala Leu
115                 120                 125                 130

CTC AAG CAG ATG ATT TAC CTG TTT CTC CAG GTT CCA GAG GCC AAC GAG      549
Leu Lys Gln Met Ile Tyr Leu Phe Leu Gln Val Pro Glu Ala Asn Glu
                135                 140                 145

GGC CTA AAG GAT GAA GTA ACC CTC TTG ACC CAA AAC ATA AGG GAC AAG      597
Gly Leu Lys Asp Glu Val Thr Leu Leu Thr Gln Asn Ile Arg Asp Lys
            150                 155                 160

GCC TAT GGA AGT GGG ACC TAC ATG GGA CAA GCA AAT CGA CTT GTG GCC      645
Ala Tyr Gly Ser Gly Thr Tyr Met Gly Gln Ala Asn Arg Leu Val Ala
        165                 170                 175
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ATG AAG GAG GTC GCC ACT GGA AGA AAC CCA AAC AAG GAT CCT CTA AAG        693
Met Lys Glu Val Ala Thr Gly Arg Asn Pro Asn Lys Asp Pro Leu Lys
    180                 185                 190

CTT GGG TAC ACT TTT GAG AGC ATC GCG CAG CTA CTT GAC ATC ACA CTA        741
Leu Gly Tyr Thr Phe Glu Ser Ile Ala Gln Leu Leu Asp Ile Thr Leu
195                 200                 205                 210

CCG GTA GGC CCA CCC GGT GAG GAT GAC AAG CCC TGG GTG CCA CTC ACA        789
Pro Val Gly Pro Pro Gly Glu Asp Asp Lys Pro Trp Val Pro Leu Thr
                215                 220                 225

AGA GTG CCG TCA CGG ATG TTG GTG CTG ACG GGA GAC GTA GAT GGC GAC        837
Arg Val Pro Ser Arg Met Leu Val Leu Thr Gly Asp Val Asp Gly Asp
            230                 235                 240

TTT GAG GTT GAA GAT TAC CTT CCC AAA ATC AAC CTC AAG TCA TCA AGT        885
Phe Glu Val Glu Asp Tyr Leu Pro Lys Ile Asn Leu Lys Ser Ser Ser
        245                 250                 255

GGA CTA CCA TAT GTA GGT CGC ACC AAA GGA GAG ACA ATT GGC GAG ATG        933
Gly Leu Pro Tyr Val Gly Arg Thr Lys Gly Glu Thr Ile Gly Glu Met
    260                 265                 270

ATA GCT ATC TCA AAC CAG TTT CTC AGA GAG CTA TCA ACA CTG TTG AAG        981
Ile Ala Ile Ser Asn Gln Phe Leu Arg Glu Leu Ser Thr Leu Leu Lys
275                 280                 285                 290

CAA GGT GCA GGG ACA AAG GGG TCA AAC AAG AAG AAG CTA CTC AGC ATG       1029
Gln Gly Ala Gly Thr Lys Gly Ser Asn Lys Lys Lys Leu Leu Ser Met
                295                 300                 305

TTA AGT GAC TAT TGG TAC TTA TCA TGC GGG CTT TTG TTT CCA AAG GCT       1077
Leu Ser Asp Tyr Trp Tyr Leu Ser Cys Gly Leu Leu Phe Pro Lys Ala
            310                 315                 320

GAA AGG TAC GAC AAA AGT ACA TGG CTC ACC AAG ACC CGG AAC ATA TGG       1125
Glu Arg Tyr Asp Lys Ser Thr Trp Leu Thr Lys Thr Arg Asn Ile Trp
        325                 330                 335

TCA GCT CCA TCC CCA ACA CAC CTC ATG ATC TCT ATG ATC ACC TGG CCC       1173
Ser Ala Pro Ser Pro Thr His Leu Met Ile Ser Met Ile Thr Trp Pro
    340                 345                 350

GTG ATG TCC AAC AGC CCA AAT AAC GTG TTG AAC ATT GAA GGG TGT CCA       1221
Val Met Ser Asn Ser Pro Asn Asn Val Leu Asn Ile Glu Gly Cys Pro
355                 360                 365                 370
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TCA CTC TAC AAA TTC AAC CCG TTC AGA GGA GGG TTG AAC AGG ATC GTC      1269
Ser Leu Tyr Lys Phe Asn Pro Phe Arg Gly Gly Leu Asn Arg Ile Val
                375                 380                 385

GAG TGG ATA TTG GCC CCG GAA GAA CCC AAG GCT CTT GTA TAT GCG GAC      1317
Glu Trp Ile Leu Ala Pro Glu Glu Pro Lys Ala Leu Val Tyr Ala Asp
            390                 395                 400

AAC ATA TAC ATT GTC CAC TCA AAC ACG TGG TAC TCA ATT GAC CTA GAG      1365
Asn Ile Tyr Ile Val His Ser Asn Thr Trp Tyr Ser Ile Asp Leu Glu
        405                 410                 415

AAG GGT GAG GCA AAC TGC ACT CGC CAA CAC ATG CAA GCC GCA ATG TAC      1413
Lys Gly Glu Ala Asn Cys Thr Arg Gln His Met Gln Ala Ala Met Tyr
    420                 425                 430

TAC ATA CTC ACC AGA GGG TGG TCA GAC AAC GGC GAC CCA ATG TTC AAT      1461
Tyr Ile Leu Thr Arg Gly Trp Ser Asp Asn Gly Asp Pro Met Phe Asn
435                 440                 445                 450

CAA ACA TGG GCC ACC TTT GCC ATG AAC ATT GCC CCT GCT CTA GTG GTG      1509
Gln Thr Trp Ala Thr Phe Ala Met Asn Ile Ala Pro Ala Leu Val Val
                455                 460                 465

GAC TCA TCG TGC CTG ATA ATG AAC CTG CAA ATT AAG ACC TAT GGT CAA      1557
Asp Ser Ser Cys Leu Ile Met Asn Leu Gln Ile Lys Thr Tyr Gly Gln
            470                 475                 480

GGC AGC GGG AAT GCA GCC ACG TTC ATC AAC AAC CAC CTC TTG AGC ACA      1605
Gly Ser Gly Asn Ala Ala Thr Phe Ile Asn Asn His Leu Leu Ser Thr
        485                 490                 495

CTA GTG CTT GAC CAG TGG AAC CTG ATG AGA CAG CCC AGA CCA GAC AGC      1653
Leu Val Leu Asp Gln Trp Asn Leu Met Arg Gln Pro Arg Pro Asp Ser
    500                 505                 510

GAG GAG TTC AAA TCA ATT GAG GAC AAG CTA GGT ATC AAC TTT AAG ATT      1701
Glu Glu Phe Lys Ser Ile Glu Asp Lys Leu Gly Ile Asn Phe Lys Ile
515                 520                 525                 530

GAG AGG TCC ATT GAT GAT ATC AGG GGC AAG CTG AGA CAG CTT GTC CTC      1749
Glu Arg Ser Ile Asp Asp Ile Arg Gly Lys Leu Arg Gln Leu Val Leu
                535                 540                 545

CTT GCA CAA CCA GGG TAC CTG AGT GGG GGG GTT GAA CCA GAA CAA TCC      1797
Leu Ala Gln Pro Gly Tyr Leu Ser Gly Gly Val Glu Pro Glu Gln Ser
            550                 555                 560
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AGC CCA ACT GTT GAG CTT GAC CTA CTA GGG TGG TCA GCT ACA TAC AGC 1845
Ser Pro Thr Val Glu Leu Asp Leu Leu Gly Trp Ser Ala Thr Tyr Ser
565 570 575

AAA GAT CTC GGG ATC TAT GTG CCG GTG CTT GAC AAG GAA CGC CTA TTT 1893
Lys Asp Leu Gly Ile Tyr Val Pro Val Leu Asp Lys Glu Arg Leu Phe
580 585 590

TGT TCT GCT GCG TAT CCC AAG GGA GTA GAG AAC AAG AGT CTC AAG TCC 1941
Cys Ser Ala Ala Tyr Pro Lys Gly Val Glu Asn Lys Ser Leu Lys Ser
595 600 605 610

AAA GTC GGG ATC GAG CAG GCA TAC AAG GTA GTC AGG TAT GAG GCG TTG 1989
Lys Val Gly Ile Glu Gln Ala Tyr Lys Val Val Arg Tyr Glu Ala Leu
615 620 625

AGG TTG GTA GGT GGT TGG AAC TAC CCA CTC CTG AAC AAA GCC TGC AAG 2037
Arg Leu Val Gly Gly Trp Asn Tyr Pro Leu Leu Asn Lys Ala Cys Lys
630 635 640

AAT AAC GCA GGC GCC GCT CGG CGG CAT CTG GAG GCC AAG GGG TTC CCA 2085
Asn Asn Ala Gly Ala Ala Arg Arg His Leu Glu Ala Lys Gly Phe Pro
645 650 655

CTC GAC GAG TTC CTA GCC GAG TGG TCT GAG CTG TCA GAG TTC GGT GAG 2133
Leu Asp Glu Phe Leu Ala Glu Trp Ser Glu Leu Ser Glu Phe Gly Glu
660 665 670

GCC TTC GAA GGC TTC AAT ATC AAG CTG ACC GTA ACA TCT GAG AGC CTA 2181
Ala Phe Glu Gly Phe Asn Ile Lys Leu Thr Val Thr Ser Glu Ser Leu
675 680 685 690

GCC GAA CTG AAC AAG CCA GTA CCC CCC AAG CCC CCA AAT GTC AAC AGA 2229
Ala Glu Leu Asn Lys Pro Val Pro Pro Lys Pro Pro Asn Val Asn Arg
695 700 705

CCA GTC AAC ACT GGG GGA CTC AAG GCA GTC AGC AAC GCC CTC AAG ACC 2277
Pro Val Asn Thr Gly Gly Leu Lys Ala Val Ser Asn Ala Leu Lys Thr
710 715 720

GGT CGG TAC AGG AAC GAA GCC GGA CTG AGT GGT CTC GTC CTT CTA GCC 2325
Gly Arg Tyr Arg Asn Glu Ala Gly Leu Ser Gly Leu Val Leu Leu Ala
725 730 735

ACA GCA AGA AGC CGT CTG CAA GAT GCA GTT AAG GCC AAG GCA GAA GCC 2373
Thr Ala Arg Ser Arg Leu Gln Asp Ala Val Lys Ala Lys Ala Glu Ala
740 745 750

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GAG AAA CTC CAC AAG TCC AAG CCA GAC GAC CCC GAT GCA GAC TGG TTC      2421
Glu Lys Leu His Lys Ser Lys Pro Asp Asp Pro Asp Ala Asp Trp Phe
755                 760                 765                 770

GAA AGA TCA GAA ACT CTG TCA GAC CTT CTG GAG AAA GCC GAC ATC GCC      2469
Glu Arg Ser Glu Thr Leu Ser Asp Leu Leu Glu Lys Ala Asp Ile Ala
                775                 780                 785

AGC AAG GTC GCC CAC TCA GCA CTC GTG GAA ACA AGC GAC GCC CTT GAA      2517
Ser Lys Val Ala His Ser Ala Leu Val Glu Thr Ser Asp Ala Leu Glu
            790                 795                 800

GCA GTT CAG TCG ACT TCC GTG TAC ACC CCC AAG TAC CCA GAA GTC AAG      2565
Ala Val Gln Ser Thr Ser Val Tyr Thr Pro Lys Tyr Pro Glu Val Lys
        805                 810                 815

AAC CCA CAG ACC GCC TCC AAC CCC GTT GTT GGG CTC CAC CTG CCC GCC      2613
Asn Pro Gln Thr Ala Ser Asn Pro Val Val Gly Leu His Leu Pro Ala
    820                 825                 830

AAG AGA GCC ACC GGT GTC CAG GCC GCT CTT CTC GGA GCA GGA ACG AGC      2661
Lys Arg Ala Thr Gly Val Gln Ala Ala Leu Leu Gly Ala Gly Thr Ser
835                 840                 845                 850

AGA CCA ATG GGG ATG GAG GCC CCA ACA CGG TCC AAG AAC GCC GTG AAA      2709
Arg Pro Met Gly Met Glu Ala Pro Thr Arg Ser Lys Asn Ala Val Lys
                855                 860                 865

ATG GCC AAA CGG CGG CAA CGC CAA AAG GAG AGC CGC TAACAGCCAT           2755
Met Ala Lys Arg Arg Gln Arg Gln Lys Glu Ser Arg
            870                 875

GATGGGAACC ACTCAAGAAG AGGACACTAA TCCCAGACCC CGTATCCCCG GCCTTCGCCT    2815

GCGGGGGCCC CC                                                        2827
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 878 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ser Asp Ile Phe Asn Ser Pro Gln Ala Arg Ser Thr Ile Ser Ala
1 5 10 15

Ala Phe Gly Ile Lys Pro Thr Ala Gly Gln Asp Val Glu Glu Leu Leu
20 25 30

Ile Pro Lys Val Trp Val Pro Pro Glu Asp Pro Leu Ala Ser Pro Ser
35 40 45

Arg Leu Ala Lys Phe Leu Arg Glu Asn Gly Tyr Lys Val Leu Gln Pro
50 55 60

Arg Ser Leu Pro Glu Asn Glu Glu Tyr Glu Thr Asp Gln Ile Leu Pro
65 70 75 80

Asp Leu Ala Trp Met Arg Gln Ile Glu Gly Ala Val Leu Lys Pro Thr
85 90 95

Leu Ser Leu Pro Ile Gly Asp Gln Glu Tyr Phe Pro Lys Tyr Tyr Pro
100 105 110

Thr His Arg Pro Ser Lys Glu Lys Pro Asn Ala Tyr Pro Pro Asp Ile
115 120 125

Ala Leu Leu Lys Gln Met Ile Tyr Leu Phe Leu Gln Val Pro Glu Ala
130 135 140

Asn Glu Gly Leu Lys Asp Glu Val Thr Leu Leu Thr Gln Asn Ile Arg
145 150 155 160

Asp Lys Ala Tyr Gly Ser Gly Thr Tyr Met Gly Gln Ala Asn Arg Leu
165 170 175

Val Ala Met Lys Glu Val Ala Thr Gly Arg Asn Pro Asn Lys Asp Pro
180 185 190

Leu Lys Leu Gly Tyr Thr Phe Glu Ser Ile Ala Gln Leu Leu Asp Ile
195 200 205

Thr Leu Pro Val Gly Pro Pro Gly Glu Asp Asp Lys Pro Trp Val Pro
210 215 220

Leu Thr Arg Val Pro Ser Arg Met Leu Val Leu Thr Gly Asp Val Asp
225 230 235 240

Gly Asp Phe Glu Val Glu Asp Tyr Leu Pro Lys Ile Asn Leu Lys Ser
245 250 255

Ser Ser Gly Leu Pro Tyr Val Gly Arg Thr Lys Gly Glu Thr Ile Gly
260 265 270

Glu Met Ile Ala Ile Ser Asn Gln Phe Leu Arg Glu Leu Ser Thr Leu
275 280 285

Leu Lys Gln Gly Ala Gly Thr Lys Gly Ser Asn Lys Lys Lys Leu Leu
290 295 300

Ser Met Leu Ser Asp Tyr Trp Tyr Leu Ser Cys Gly Leu Leu Phe Pro
305 310 315 320

Lys Ala Glu Arg Tyr Asp Lys Ser Thr Trp Leu Thr Lys Thr Arg Asn
325 330 335

Ile Trp Ser Ala Pro Ser Pro Thr His Leu Met Ile Ser Met Ile Thr
340 345 350

Trp Pro Val Met Ser Asn Ser Pro Asn Asn Val Leu Asn Ile Glu Gly
355 360 365

Cys Pro Ser Leu Tyr Lys Phe Asn Pro Phe Arg Gly Gly Leu Asn Arg
370 375 380

Ile Val Glu Trp Ile Leu Ala Pro Glu Glu Pro Lys Ala Leu Val Tyr
385 390 395 400

Ala Asp Asn Ile Tyr Ile Val His Ser Asn Thr Trp Tyr Ser Ile Asp
405 410 415

Leu Glu Lys Gly Glu Ala Asn Cys Thr Arg Gln His Met Gln Ala Ala
420 425 430

Met Tyr Tyr Ile Leu Thr Arg Gly Trp Ser Asp Asn Gly Asp Pro Met
435 440 445

Phe Asn Gln Thr Trp Ala Thr Phe Ala Met Asn Ile Ala Pro Ala Leu
450 455 460

Val Val Asp Ser Ser Cys Leu Ile Met Asn Leu Gln Ile Lys Thr Tyr
465 470 475 480

Gly Gln Gly Ser Gly Asn Ala Ala Thr Phe Ile Asn Asn His Leu Leu
485 490 495

Ser Thr Leu Val Leu Asp Gln Trp Asn Leu Met Arg Gln Pro Arg Pro
500 505 510

Asp Ser Glu Glu Phe Lys Ser Ile Glu Asp Lys Leu Gly Ile Asn Phe
515 520 525

Lys Ile Glu Arg Ser Ile Asp Asp Ile Arg Gly Lys Leu Arg Gln Leu
530 535 540

Val Leu Leu Ala Gln Pro Gly Tyr Leu Ser Gly Gly Val Glu Pro Glu
545 550 555 560

Gln Ser Ser Pro Thr Val Glu Leu Asp Leu Leu Gly Trp Ser Ala Thr
565 570 575

Tyr Ser Lys Asp Leu Gly Ile Tyr Val Pro Val Leu Asp Lys Glu Arg
580 585 590

Leu Phe Cys Ser Ala Ala Tyr Pro Lys Gly Val Glu Asn Lys Ser Leu
595 600 605

Lys Ser Lys Val Gly Ile Glu Gln Ala Tyr Lys Val Val Arg Tyr Glu
610 615 620

Ala Leu Arg Leu Val Gly Gly Trp Asn Tyr Pro Leu Leu Asn Lys Ala
625 630 635 640

Cys Lys Asn Asn Ala Gly Ala Ala Arg Arg His Leu Glu Ala Lys Gly
645 650 655

Phe Pro Leu Asp Glu Phe Leu Ala Glu Trp Ser Glu Leu Ser Glu Phe
660 665 670

Gly Glu Ala Phe Glu Gly Phe Asn Ile Lys Leu Thr Val Thr Ser Glu
675 680 685

Ser Leu Ala Glu Leu Asn Lys Pro Val Pro Pro Lys Pro Pro Asn Val
690 695 700

Asn Arg Pro Val Asn Thr Gly Gly Leu Lys Ala Val Ser Asn Ala Leu
705 710 715 720

Lys Thr Gly Arg Tyr Arg Asn Glu Ala Gly Leu Ser Gly Leu Val Leu
725 730 735

Leu Ala Thr Ala Arg Ser Arg Leu Gln Asp Ala Val Lys Ala Lys Ala
740 745 750

Glu Ala Glu Lys Leu His Lys Ser Lys Pro Asp Asp Pro Asp Ala Asp
755 760 765

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Trp Phe Glu Arg Ser Glu Thr Leu Ser Asp Leu Leu Glu Lys Ala Asp
    770                 775                 780

Ile Ala Ser Lys Val Ala His Ser Ala Leu Val Glu Thr Ser Asp Ala
785                 790                 795                 800

Leu Glu Ala Val Gln Ser Thr Ser Val Tyr Thr Pro Lys Tyr Pro Glu
                805                 810                 815

Val Lys Asn Pro Gln Thr Ala Ser Asn Pro Val Val Gly Leu His Leu
            820                 825                 830

Pro Ala Lys Arg Ala Thr Gly Val Gln Ala Ala Leu Leu Gly Ala Gly
        835                 840                 845

Thr Ser Arg Pro Met Gly Met Glu Ala Pro Thr Arg Ser Lys Asn Ala
    850                 855                 860

Val Lys Met Ala Lys Arg Arg Gln Arg Gln Lys Glu Ser Arg
865                 870                 875
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3261 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:97..531

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
GGATACGATC GGTCTGACCC CGGGGGAGTC ACCCGGGGAC AGGCCGTCAA GGCCTTGTTC      60

CAGGATGGGA CTCCTCCTTC TACAACGCTA TCATTG ATG GTT AGT AGA GAT CAG       114
                                        Met Val Ser Arg Asp Gln
                                         1               5
```

ACA AAC GAT CGC AGC GAT GAC AAA CCT GCA AGA TCA AAC CCA ACA GAT 162
Thr Asn Asp Arg Ser Asp Asp Lys Pro Ala Arg Ser Asn Pro Thr Asp
10 15 20

TGT TCC GTT CAT ACG GAG CCT TCT GAT GCC AAC AAC CGG ACC GGC GTC 210
Cys Ser Val His Thr Glu Pro Ser Asp Ala Asn Asn Arg Thr Gly Val
25 30 35

CAT TCC GGA CGA CAC CCT GGA GAA GCA CAC TCT CAG GTC AGA GAC CTC 258
His Ser Gly Arg His Pro Gly Glu Ala His Ser Gln Val Arg Asp Leu
40 45 50

GAC CTA CAA TTT GAC TGT GGG GGA CAC AGG GTC AGG GCT AAT TGT CTT 306
Asp Leu Gln Phe Asp Cys Gly Gly His Arg Val Arg Ala Asn Cys Leu
55 60 65 70

TTT CCC TGG ATT CCC TGG CTC AAT TGT GGG TGC TCA CTA CAC ACT GCA 354
Phe Pro Trp Ile Pro Trp Leu Asn Cys Gly Cys Ser Leu His Thr Ala
75 80 85

GGG CAA TGG GAA CTA CAA GTT CGA TCA GAT GCT CCT GAC TGC CCA GAA 402
Gly Gln Trp Glu Leu Gln Val Arg Ser Asp Ala Pro Asp Cys Pro Glu
90 95 100

CCT ACC GGC CAG TTA CAA CTA CTG CAG GCT AGT GAG TCG GAG TCT CAC 450
Pro Thr Gly Gln Leu Gln Leu Leu Gln Ala Ser Glu Ser Glu Ser His
105 110 115

AGT GAG GTC AAG CAC ACT TCC TGG TGG CGT TTA TGC ACT AAA CGG CAC 498
Ser Glu Val Lys His Thr Ser Trp Trp Arg Leu Cys Thr Lys Arg His
120 125 130

CAT AAA CGC CGT GAC CTT CCA AGG AAG CCT GAG TGAACTGACA GATGTTAGCT 551
His Lys Arg Arg Asp Leu Pro Arg Lys Pro Glu
135 140 145

ACAATGGGTT GATGTCTGCA ACAGCCAACA TCAACGACAA AATTGGGAAC GTCCTAGTAG 611

GGGAAGGGGT CACCGTCCTC AGCTTACCCA CATCATATGA TCTTGGGTAT GTGAGGCTTG 671

GTGACCCCAT TCCCGCAATA GGGCTTGACC CAAAAATGGT AGCCACATGT GACAGCAGTG 731

ACAGGCCCAG AGTCTACACC ATAACTGCAG CCGATGATTA CCAATTCTCA TCACAGTACC 791

AACCAGGTGG GGTAACAATC ACACTGTTCT CAGCCAACAT TGATGCCATC ACAAGCCTCA 851

GCGTTGGGGG AGAGCTCGTG TTTCAAACAA GCGTCCACGG CCTTGTACTG GGCGCCACCA 911

TCTACCTCAT AGGCTTTGAT GGGACAACGG TAATCACCAG GGCTGTGGCC GCAAACAATG 971

GGCTGACGAC CGGCACCGAC AACCTTATGC CATTCAATCT TGTGATTCCA ACAAACGAGA 1031

TAACCCAGCC AATCACATCC ATCAAACTGG AGATAGTGAC CTCCAAAAGT GGTGGTCAGG 1091

CAGGGGATCA GATGTCATGG TCGGCAAGAG GGAGCCTAGC AGTGACGATC CATGGTGGCA 1151

ACTATCCAGG GGCCCTCCGT CCCGTCACGC TAGTGGCCTA CGAAAGAGTG GCAACAGGAT 1211

CCGTCGTTAC GGTCGCTGGG GTGAGCAACT TCGAGCTGAT CCCAAATCCT GAACTAGCAA 1271

AGAACCTGGT TACAGAATAC GGCCGATTTG ACCCAGGAGC CATGAACTAC ACAAAATTGA 1331

TACTGAGTGA GAGGGACCGT CTTGGCATCA AGACCGTCTG GCCAACAAGG GAGTACACTG 1391

ACTTTCGTGA ATACTTCATG GAGGTGGCCG ACCTCAACTC TCCCCTGAAG ATTGCAGGAG 1451

CATTCGGCTT CAAAGACATA ATCCGGGCCA TAAGGAGGAT AGCTGTGCCG GTGGTCTCCA 1511

CATTGTTCCC ACCTGCCGCT CCCCTAGCCC ATGCAATTGG GGAAGGTGTA GACTACCTGC 1571

TGGGCGATGA GGCACAGGCT GCTTCAGGAA CTGCTCGAGC CGCGTCAGGA AAAGCAAGAG 1631

CTGCCTCAGG CCGCATAAGG CAGCTGACTC TCGCCGCCGA CAAGGGGTAC GAGGTAGTCG 1691

CGAATCTATT CCAGGTGCCC CAGAATCCCG TAGTCGACGG GATTCTTGCT TCACCTGGGG 1751

TACTCCGCGG TGCACACAAC CTCGACTGCG TGTTAAGAGA GGGTGCCACG CTATTCCCTG 1811

TGGTTATTAC GACAGTGGAA GACGCCATGA CACCCAAAGC ATTGAACAGC AAAATGTTTG 1871

CTGTCATTGA AGGCGTGCGA GAAGACCTCC AACCTCCATC TCAAAGAGGA TCCTTCATAC 1931

GAACTCTCTC TGGACACAGA GTCTATGGAT ATGCTCCAGA TGGGGTACTT CCACTGGAGA 1991

CTGGGAGAGA CTACACCGTT GTCCCAATAG ATGATGTCTG GGACGACAGC ATTATGCTGT 2051

CCAAAGATCC CATACCTCCT ATTGTGGGAA ACAGTGGAAA TCTAGCCATA GCTTACATGG 2111

ATGTGTTTCG ACCCAAAGTC CCAATCCATG TGGCTATGAC GGGAGCCCTC AATGCTTGTG 2171

GCGAGATTGA GAAAGTAAGC TTTAGAAGCA CCAAGCTCGC CACTGCACAC CGACTTGGCC 2231

TTAGGTTGGC TGGTCCCGGA GCATTCGATG TAAACACCGG GCCCAACTGG GCAACGTTCA 2291

TCAAACGTTT CCCTCACAAT CCACGCGACT GGGACAGGCT CCCCTACCTC AACCTACCAT 2351

ACCTTCCACC CAATGCAGGA CGCCAGTACC ACCTTGCCAT GGCTGCATCA GAGTTCAAAG 2411

AGACCCCCGA ACTCGAGAGT GCCGTCAGAG CAATGGAAGC AGCAGCCAAC GTGGACCCAC 2471

TATTCCAATC TGCACTCAGT GTGTTCATGT GGCTGGAAGA GAATGGGATT GTGACTGACA 2531

TGGCCAACTT CGCACTCAGC GACCCGAACG CCCATCGGAT GCGAAATTTT CTTGCAAACG 2591

CACCACAAGC AGGCAGCAAG TCGCAAAGGG CCAAGTACGG GACAGCAGGC TACGGAGTGG 2651

AGGCTCGGGG CCCCACACCA GAGGAAGCAC AGAGGGAAAA AGACACACGG ATCTCAAAGA 2711

AGATGGAGAC CATGGGCATC TACTTTGCAA CACCAGAATG GGTAGCACTC AATGGGCACC 2771

GAGGGCCAAG CCCCGGCCAG CTAAAGTACT GGCAGAACAC ACGAGAAATA CCGGACCCAA 2831

ACGAGGACTA TCTAGACTAC GTGCATGCAG AGAAGAGCCG GTTGGCATCA GAAGAACAAA 2891

TCCTAAGGGC AGCTACGTCG ATCTACGGGG CTCCAGGACA GGCAGAGCCA CCCCAAGCTT 2951

TCATAGACGA AGTTGCCAAA GTCTATGAAA TCAACCATGG ACGTGGCCCA AACCAAGAAC 3011

AGATGAAAGA TCTGCTCTTG ACTGCGATGG AGATGAAGCA TCGCAATCCC AGGCGGGCTC 3071

TACCAAAGCC CAAGCCAAAA CCCAATGCTC CAACACAGAG ACCCCCTGGT CGGCTGGGCC 3131

GCTGGATCAG GACCGTCTCT GATGAGGACC TTGAGTGAGG CTCCTGGGAG TCTCCCGACA 3191

CCACCCGCGC AGGTGTGGAC ACCAATTCGG CCTTACAACA TCCCAAATTG GATCCGTTCG 3251

CGGGTCCCCT 3261

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 145 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Val Ser Arg Asp Gln Thr Asn Asp Arg Ser Asp Asp Lys Pro Ala
1 5 10 15

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Arg Ser Asn Pro Thr Asp Cys Ser Val His Thr Glu Pro Ser Asp Ala
            20                  25                  30

Asn Asn Arg Thr Gly Val His Ser Gly Arg His Pro Gly Glu Ala His
        35                  40                  45

Ser Gln Val Arg Asp Leu Asp Leu Gln Phe Asp Cys Gly Gly His Arg
    50                  55                  60

Val Arg Ala Asn Cys Leu Phe Pro Trp Ile Pro Trp Leu Asn Cys Gly
65                  70                  75                  80

Cys Ser Leu His Thr Ala Gly Gln Trp Glu Leu Gln Val Arg Ser Asp
                85                  90                  95

Ala Pro Asp Cys Pro Glu Pro Thr Gly Gln Leu Gln Leu Leu Gln Ala
            100                 105                 110

Ser Glu Ser Glu Ser His Ser Glu Val Lys His Thr Ser Trp Trp Arg
        115                 120                 125

Leu Cys Thr Lys Arg His His Lys Arg Arg Asp Leu Pro Arg Lys Pro
    130                 135                 140

Glu
145
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 3261 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
- (A) NAME/KEY: CDS
- (B) LOCATION:131..3166

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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GGATACGATC GGTCTGACCC CGGGGGAGTC ACCCGGGGAC AGGCCGTCAA GGCCTTGTTC      60
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CAGGATGGGA CTCCTCCTTC TACAACGCTA TCATTGATGG TTAGTAGAGA TCAGACAAAC      120

GATCGCAGCG ATG ACA AAC CTG CAA GAT CAA ACC CAA CAG ATT GTT CCG        169
           Met Thr Asn Leu Gln Asp Gln Thr Gln Gln Ile Val Pro
             1               5                  10

TTC ATA CGG AGC CTT CTG ATG CCA ACA ACC GGA CCG GCG TCC ATT CCG       217
Phe Ile Arg Ser Leu Leu Met Pro Thr Thr Gly Pro Ala Ser Ile Pro
     15                  20                  25

GAC GAC ACC CTG GAG AAG CAC ACT CTC AGG TCA GAG ACC TCG ACC TAC       265
Asp Asp Thr Leu Glu Lys His Thr Leu Arg Ser Glu Thr Ser Thr Tyr
 30                  35                  40                  45

AAT TTG ACT GTG GGG GAC ACA GGG TCA GGG CTA ATT GTC TTT TTC CCT       313
Asn Leu Thr Val Gly Asp Thr Gly Ser Gly Leu Ile Val Phe Phe Pro
                 50                  55                  60

GGA TTC CCT GGC TCA ATT GTG GGT GCT CAC TAC ACA CTG CAG GGC AAT       361
Gly Phe Pro Gly Ser Ile Val Gly Ala His Tyr Thr Leu Gln Gly Asn
             65                  70                  75

GGG AAC TAC AAG TTC GAT CAG ATG CTC CTG ACT GCC CAG AAC CTA CCG       409
Gly Asn Tyr Lys Phe Asp Gln Met Leu Leu Thr Ala Gln Asn Leu Pro
         80                  85                  90

GCC AGT TAC AAC TAC TGC AGG CTA GTG AGT CGG AGT CTC ACA GTG AGG       457
Ala Ser Tyr Asn Tyr Cys Arg Leu Val Ser Arg Ser Leu Thr Val Arg
     95                 100                 105

TCA AGC ACA CTT CCT GGT GGC GTT TAT GCA CTA AAC GGC ACC ATA AAC       505
Ser Ser Thr Leu Pro Gly Gly Val Tyr Ala Leu Asn Gly Thr Ile Asn
110                 115                 120                 125

GCC GTG ACC TTC CAA GGA AGC CTG AGT GAA CTG ACA GAT GTT AGC TAC       553
Ala Val Thr Phe Gln Gly Ser Leu Ser Glu Leu Thr Asp Val Ser Tyr
                130                 135                 140

AAT GGG TTG ATG TCT GCA ACA GCC AAC ATC AAC GAC AAA ATT GGG AAC       601
Asn Gly Leu Met Ser Ala Thr Ala Asn Ile Asn Asp Lys Ile Gly Asn
            145                 150                 155

GTC CTA GTA GGG GAA GGG GTC ACC GTC CTC AGC TTA CCC ACA TCA TAT       649
Val Leu Val Gly Glu Gly Val Thr Val Leu Ser Leu Pro Thr Ser Tyr
        160                 165                 170

GAT CTT GGG TAT GTG AGG CTT GGT GAC CCC ATT CCC GCA ATA GGG CTT       697
```

Asp Leu Gly Tyr Val Arg Leu Gly Asp Pro Ile Pro Ala Ile Gly Leu
175 180 185

GAC CCA AAA ATG GTA GCC ACA TGT GAC AGC AGT GAC AGG CCC AGA GTC 745
Asp Pro Lys Met Val Ala Thr Cys Asp Ser Ser Asp Arg Pro Arg Val
190 195 200 205

TAC ACC ATA ACT GCA GCC GAT GAT TAC CAA TTC TCA TCA CAG TAC CAA 793
Tyr Thr Ile Thr Ala Ala Asp Asp Tyr Gln Phe Ser Ser Gln Tyr Gln
210 215 220

CCA GGT GGG GTA ACA ATC ACA CTG TTC TCA GCC AAC ATT GAT GCC ATC 841
Pro Gly Gly Val Thr Ile Thr Leu Phe Ser Ala Asn Ile Asp Ala Ile
225 230 235

ACA AGC CTC AGC GTT GGG GGA GAG CTC GTG TTT CAA ACA AGC GTC CAC 889
Thr Ser Leu Ser Val Gly Gly Glu Leu Val Phe Gln Thr Ser Val His
240 245 250

GGC CTT GTA CTG GGC GCC ACC ATC TAC CTC ATA GGC TTT GAT GGG ACA 937
Gly Leu Val Leu Gly Ala Thr Ile Tyr Leu Ile Gly Phe Asp Gly Thr
255 260 265

ACG GTA ATC ACC AGG GCT GTG GCC GCA AAC AAT GGG CTG ACG ACC GGC 985
Thr Val Ile Thr Arg Ala Val Ala Ala Asn Asn Gly Leu Thr Thr Gly
270 275 280 285

ACC GAC AAC CTT ATG CCA TTC AAT CTT GTG ATT CCA ACA AAC GAG ATA 1033
Thr Asp Asn Leu Met Pro Phe Asn Leu Val Ile Pro Thr Asn Glu Ile
290 295 300

ACC CAG CCA ATC ACA TCC ATC AAA CTG GAG ATA GTG ACC TCC AAA AGT 1081
Thr Gln Pro Ile Thr Ser Ile Lys Leu Glu Ile Val Thr Ser Lys Ser
305 310 315

GGT GGT CAG GCA GGG GAT CAG ATG TCA TGG TCG GCA AGA GGG AGC CTA 1129
Gly Gly Gln Ala Gly Asp Gln Met Ser Trp Ser Ala Arg Gly Ser Leu
320 325 330

GCA GTG ACG ATC CAT GGT GGC AAC TAT CCA GGG GCC CTC CGT CCC GTC 1177
Ala Val Thr Ile His Gly Gly Asn Tyr Pro Gly Ala Leu Arg Pro Val
335 340 345

ACG CTA GTG GCC TAC GAA AGA GTG GCA ACA GGA TCC GTC GTT ACG GTC 1225
Thr Leu Val Ala Tyr Glu Arg Val Ala Thr Gly Ser Val Val Thr Val
350 355 360 365

GCT GGG GTG AGC AAC TTC GAG CTG ATC CCA AAT CCT GAA CTA GCA AAG 1273

Ala Gly Val Ser Asn Phe Glu Leu Ile Pro Asn Pro Glu Leu Ala Lys
370 375 380

AAC CTG GTT ACA GAA TAC GGC CGA TTT GAC CCA GGA GCC ATG AAC TAC 1321
Asn Leu Val Thr Glu Tyr Gly Arg Phe Asp Pro Gly Ala Met Asn Tyr
385 390 395

ACA AAA TTG ATA CTG AGT GAG AGG GAC CGT CTT GGC ATC AAG ACC GTC 1369
Thr Lys Leu Ile Leu Ser Glu Arg Asp Arg Leu Gly Ile Lys Thr Val
400 405 410

TGG CCA ACA AGG GAG TAC ACT GAC TTT CGT GAA TAC TTC ATG GAG GTG 1417
Trp Pro Thr Arg Glu Tyr Thr Asp Phe Arg Glu Tyr Phe Met Glu Val
415 420 425

GCC GAC CTC AAC TCT CCC CTG AAG ATT GCA GGA GCA TTC GGC TTC AAA 1465
Ala Asp Leu Asn Ser Pro Leu Lys Ile Ala Gly Ala Phe Gly Phe Lys
430 435 440 445

GAC ATA ATC CGG GCC ATA AGG AGG ATA GCT GTG CCG GTG GTC TCC ACA 1513
Asp Ile Ile Arg Ala Ile Arg Arg Ile Ala Val Pro Val Val Ser Thr
450 455 460

TTG TTC CCA CCT GCC GCT CCC CTA GCC CAT GCA ATT GGG GAA GGT GTA 1561
Leu Phe Pro Pro Ala Ala Pro Leu Ala His Ala Ile Gly Glu Gly Val
465 470 475

GAC TAC CTG CTG GGC GAT GAG GCA CAG GCT GCT TCA GGA ACT GCT CGA 1609
Asp Tyr Leu Leu Gly Asp Glu Ala Gln Ala Ala Ser Gly Thr Ala Arg
480 485 490

GCC GCG TCA GGA AAA GCA AGA GCT GCC TCA GGC CGC ATA AGG CAG CTG 1657
Ala Ala Ser Gly Lys Ala Arg Ala Ala Ser Gly Arg Ile Arg Gln Leu
495 500 505

ACT CTC GCC GCC GAC AAG GGG TAC GAG GTA GTC GCG AAT CTA TTC CAG 1705
Thr Leu Ala Ala Asp Lys Gly Tyr Glu Val Val Ala Asn Leu Phe Gln
510 515 520 525

GTG CCC CAG AAT CCC GTA GTC GAC GGG ATT CTT GCT TCA CCT GGG GTA 1753
Val Pro Gln Asn Pro Val Val Asp Gly Ile Leu Ala Ser Pro Gly Val
530 535 540

CTC CGC GGT GCA CAC AAC CTC GAC TGC GTG TTA AGA GAG GGT GCC ACG 1801
Leu Arg Gly Ala His Asn Leu Asp Cys Val Leu Arg Glu Gly Ala Thr
545 550 555

CTA TTC CCT GTG GTT ATT ACG ACA GTG GAA GAC GCC ATG ACA CCC AAA 1849

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Leu Phe Pro Val Val Ile Thr Thr Val Glu Asp Ala Met Thr Pro Lys
        560                 565                 570

GCA TTG AAC AGC AAA ATG TTT GCT GTC ATT GAA GGC GTG CGA GAA GAC      1897
Ala Leu Asn Ser Lys Met Phe Ala Val Ile Glu Gly Val Arg Glu Asp
    575                 580                 585

CTC CAA CCT CCA TCT CAA AGA GGA TCC TTC ATA CGA ACT CTC TCT GGA      1945
Leu Gln Pro Pro Ser Gln Arg Gly Ser Phe Ile Arg Thr Leu Ser Gly
590                 595                 600                 605

CAC AGA GTC TAT GGA TAT GCT CCA GAT GGG GTA CTT CCA CTG GAG ACT      1993
His Arg Val Tyr Gly Tyr Ala Pro Asp Gly Val Leu Pro Leu Glu Thr
                610                 615                 620

GGG AGA GAC TAC ACC GTT GTC CCA ATA GAT GAT GTC TGG GAC GAC AGC      2041
Gly Arg Asp Tyr Thr Val Val Pro Ile Asp Asp Val Trp Asp Asp Ser
            625                 630                 635

ATT ATG CTG TCC AAA GAT CCC ATA CCT CCT ATT GTG GGA AAC AGT GGA      2089
Ile Met Leu Ser Lys Asp Pro Ile Pro Pro Ile Val Gly Asn Ser Gly
        640                 645                 650

AAT CTA GCC ATA GCT TAC ATG GAT GTG TTT CGA CCC AAA GTC CCA ATC      2137
Asn Leu Ala Ile Ala Tyr Met Asp Val Phe Arg Pro Lys Val Pro Ile
    655                 660                 665

CAT GTG GCT ATG ACG GGA GCC CTC AAT GCT TGT GGC GAG ATT GAG AAA      2185
His Val Ala Met Thr Gly Ala Leu Asn Ala Cys Gly Glu Ile Glu Lys
670                 675                 680                 685

GTA AGC TTT AGA AGC ACC AAG CTC GCC ACT GCA CAC CGA CTT GGC CTT      2233
Val Ser Phe Arg Ser Thr Lys Leu Ala Thr Ala His Arg Leu Gly Leu
                690                 695                 700

AGG TTG GCT GGT CCC GGA GCA TTC GAT GTA AAC ACC GGG CCC AAC TGG      2281
Arg Leu Ala Gly Pro Gly Ala Phe Asp Val Asn Thr Gly Pro Asn Trp
            705                 710                 715

GCA ACG TTC ATC AAA CGT TTC CCT CAC AAT CCA CGC GAC TGG GAC AGG      2329
Ala Thr Phe Ile Lys Arg Phe Pro His Asn Pro Arg Asp Trp Asp Arg
        720                 725                 730

CTC CCC TAC CTC AAC CTA CCA TAC CTT CCA CCC AAT GCA GGA CGC CAG      2377
Leu Pro Tyr Leu Asn Leu Pro Tyr Leu Pro Pro Asn Ala Gly Arg Gln
    735                 740                 745

TAC CAC CTT GCC ATG GCT GCA TCA GAG TTC AAA GAG ACC CCC GAA CTC      2425
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Tyr His Leu Ala Met Ala Ala Ser Glu Phe Lys Glu Thr Pro Glu Leu
750                 755                 760                 765

GAG AGT GCC GTC AGA GCA ATG GAA GCA GCC AAC GTG GAC CCA CTA      2473
Glu Ser Ala Val Arg Ala Met Glu Ala Ala Ala Asn Val Asp Pro Leu
                770                 775                 780

TTC CAA TCT GCA CTC AGT GTG TTC ATG TGG CTG GAA GAG AAT GGG ATT  2521
Phe Gln Ser Ala Leu Ser Val Phe Met Trp Leu Glu Glu Asn Gly Ile
            785                 790                 795

GTG ACT GAC ATG GCC AAC TTC GCA CTC AGC GAC CCG AAC GCC CAT CGG  2569
Val Thr Asp Met Ala Asn Phe Ala Leu Ser Asp Pro Asn Ala His Arg
        800                 805                 810

ATG CGA AAT TTT CTT GCA AAC GCA CCA CAA GCA GGC AGC AAG TCG CAA  2617
Met Arg Asn Phe Leu Ala Asn Ala Pro Gln Ala Gly Ser Lys Ser Gln
    815                 820                 825

AGG GCC AAG TAC GGG ACA GCA GGC TAC GGA GTG GAG GCT CGG GGC CCC  2665
Arg Ala Lys Tyr Gly Thr Ala Gly Tyr Gly Val Glu Ala Arg Gly Pro
830                 835                 840                 845

ACA CCA GAG GAA GCA CAG AGG GAA AAA GAC ACA CGG ATC TCA AAG AAG  2713
Thr Pro Glu Glu Ala Gln Arg Glu Lys Asp Thr Arg Ile Ser Lys Lys
                850                 855                 860

ATG GAG ACC ATG GGC ATC TAC TTT GCA ACA CCA GAA TGG GTA GCA CTC  2761
Met Glu Thr Met Gly Ile Tyr Phe Ala Thr Pro Glu Trp Val Ala Leu
            865                 870                 875

AAT GGG CAC CGA GGG CCA AGC CCC GGC CAG CTA AAG TAC TGG CAG AAC  2809
Asn Gly His Arg Gly Pro Ser Pro Gly Gln Leu Lys Tyr Trp Gln Asn
        880                 885                 890

ACA CGA GAA ATA CCG GAC CCA AAC GAG GAC TAT CTA GAC TAC GTG CAT  2857
Thr Arg Glu Ile Pro Asp Pro Asn Glu Asp Tyr Leu Asp Tyr Val His
    895                 900                 905

GCA GAG AAG AGC CGG TTG GCA TCA GAA GAA CAA ATC CTA AGG GCA GCT  2905
Ala Glu Lys Ser Arg Leu Ala Ser Glu Glu Gln Ile Leu Arg Ala Ala
910                 915                 920                 925

ACG TCG ATC TAC GGG GCT CCA GGA CAG GCA GAG CCA CCC CAA GCT TTC  2953
Thr Ser Ile Tyr Gly Ala Pro Gly Gln Ala Glu Pro Pro Gln Ala Phe
                930                 935                 940

ATA GAC GAA GTT GCC AAA GTC TAT GAA ATC AAC CAT GGA CGT GGC CCA  3001
```

Ile Asp Glu Val Ala Lys Val Tyr Glu Ile Asn His Gly Arg Gly Pro
945 950 955

AAC CAA GAA CAG ATG AAA GAT CTG CTC TTG ACT GCG ATG GAG ATG AAG 3049
Asn Gln Glu Gln Met Lys Asp Leu Leu Leu Thr Ala Met Glu Met Lys
960 965 970

CAT CGC AAT CCC AGG CGG GCT CTA CCA AAG CCC AAG CCA AAA CCC AAT 3097
His Arg Asn Pro Arg Arg Ala Leu Pro Lys Pro Lys Pro Lys Pro Asn
975 980 985

GCT CCA ACA CAG AGA CCC CCT GGT CGG CTG GGC CGC TGG ATC AGG ACC 3145
Ala Pro Thr Gln Arg Pro Pro Gly Arg Leu Gly Arg Trp Ile Arg Thr
990 995 1000 1005

GTC TCT GAT GAG GAC CTT GAG TGAGGCTCCT GGGAGTCTCC CGACACCACC 3196
Val Ser Asp Glu Asp Leu Glu
1010

CGCGCAGGTG TGGACACCAA TTCGGCCTTA CAACATCCCA AATTGGATCC GTTCGCGGGT 3256

CCCCT 3261

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1012 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Thr Asn Leu Gln Asp Gln Thr Gln Gln Ile Val Pro Phe Ile Arg
1 5 10 15

Ser Leu Leu Met Pro Thr Thr Gly Pro Ala Ser Ile Pro Asp Asp Thr
20 25 30

Leu Glu Lys His Thr Leu Arg Ser Glu Thr Ser Thr Tyr Asn Leu Thr
35 40 45

Val Gly Asp Thr Gly Ser Gly Leu Ile Val Phe Phe Pro Gly Phe Pro
50 55 60

Gly Ser Ile Val Gly Ala His Tyr Thr Leu Gln Gly Asn Gly Asn Tyr

65 70 75 80

Lys Phe Asp Gln Met Leu Leu Thr Ala Gln Asn Leu Pro Ala Ser Tyr
85 90 95

Asn Tyr Cys Arg Leu Val Ser Arg Ser Leu Thr Val Arg Ser Ser Thr
100 105 110

Leu Pro Gly Gly Val Tyr Ala Leu Asn Gly Thr Ile Asn Ala Val Thr
115 120 125

Phe Gln Gly Ser Leu Ser Glu Leu Thr Asp Val Ser Tyr Asn Gly Leu
130 135 140

Met Ser Ala Thr Ala Asn Ile Asn Asp Lys Ile Gly Asn Val Leu Val
145 150 155 160

Gly Glu Gly Val Thr Val Leu Ser Leu Pro Thr Ser Tyr Asp Leu Gly
165 170 175

Tyr Val Arg Leu Gly Asp Pro Ile Pro Ala Ile Gly Leu Asp Pro Lys
180 185 190

Met Val Ala Thr Cys Asp Ser Ser Asp Arg Pro Arg Val Tyr Thr Ile
195 200 205

Thr Ala Ala Asp Asp Tyr Gln Phe Ser Ser Gln Tyr Gln Pro Gly Gly
210 215 220

Val Thr Ile Thr Leu Phe Ser Ala Asn Ile Asp Ala Ile Thr Ser Leu
225 230 235 240

Ser Val Gly Gly Glu Leu Val Phe Gln Thr Ser Val His Gly Leu Val
245 250 255

Leu Gly Ala Thr Ile Tyr Leu Ile Gly Phe Asp Gly Thr Thr Val Ile
260 265 270

Thr Arg Ala Val Ala Ala Asn Asn Gly Leu Thr Thr Gly Thr Asp Asn
275 280 285

Leu Met Pro Phe Asn Leu Val Ile Pro Thr Asn Glu Ile Thr Gln Pro
290 295 300

Ile Thr Ser Ile Lys Leu Glu Ile Val Thr Ser Lys Ser Gly Gly Gln
305 310 315 320

Ala Gly Asp Gln Met Ser Trp Ser Ala Arg Gly Ser Leu Ala Val Thr

325 330 335

Ile His Gly Gly Asn Tyr Pro Gly Ala Leu Arg Pro Val Thr Leu Val
340 345 350

Ala Tyr Glu Arg Val Ala Thr Gly Ser Val Val Thr Val Ala Gly Val
355 360 365

Ser Asn Phe Glu Leu Ile Pro Asn Pro Glu Leu Ala Lys Asn Leu Val
370 375 380

Thr Glu Tyr Gly Arg Phe Asp Pro Gly Ala Met Asn Tyr Thr Lys Leu
385 390 395 400

Ile Leu Ser Glu Arg Asp Arg Leu Gly Ile Lys Thr Val Trp Pro Thr
405 410 415

Arg Glu Tyr Thr Asp Phe Arg Glu Tyr Phe Met Glu Val Ala Asp Leu
420 425 430

Asn Ser Pro Leu Lys Ile Ala Gly Ala Phe Gly Phe Lys Asp Ile Ile
435 440 445

Arg Ala Ile Arg Arg Ile Ala Val Pro Val Val Ser Thr Leu Phe Pro
450 455 460

Pro Ala Ala Pro Leu Ala His Ala Ile Gly Glu Gly Val Asp Tyr Leu
465 470 475 480

Leu Gly Asp Glu Ala Gln Ala Ala Ser Gly Thr Ala Arg Ala Ala Ser
485 490 495

Gly Lys Ala Arg Ala Ala Ser Gly Arg Ile Arg Gln Leu Thr Leu Ala
500 505 510

Ala Asp Lys Gly Tyr Glu Val Val Ala Asn Leu Phe Gln Val Pro Gln
515 520 525

Asn Pro Val Val Asp Gly Ile Leu Ala Ser Pro Gly Val Leu Arg Gly
530 535 540

Ala His Asn Leu Asp Cys Val Leu Arg Glu Gly Ala Thr Leu Phe Pro
545 550 555 560

Val Val Ile Thr Thr Val Glu Asp Ala Met Thr Pro Lys Ala Leu Asn
565 570 575

Ser Lys Met Phe Ala Val Ile Glu Gly Val Arg Glu Asp Leu Gln Pro

580 585 590

Pro Ser Gln Arg Gly Ser Phe Ile Arg Thr Leu Ser Gly His Arg Val
595 600 605

Tyr Gly Tyr Ala Pro Asp Gly Val Leu Pro Leu Glu Thr Gly Arg Asp
610 615 620

Tyr Thr Val Val Pro Ile Asp Asp Val Trp Asp Asp Ser Ile Met Leu
625 630 635 640

Ser Lys Asp Pro Ile Pro Pro Ile Val Gly Asn Ser Gly Asn Leu Ala
645 650 655

Ile Ala Tyr Met Asp Val Phe Arg Pro Lys Val Pro Ile His Val Ala
660 665 670

Met Thr Gly Ala Leu Asn Ala Cys Gly Glu Ile Glu Lys Val Ser Phe
675 680 685

Arg Ser Thr Lys Leu Ala Thr Ala His Arg Leu Gly Leu Arg Leu Ala
690 695 700

Gly Pro Gly Ala Phe Asp Val Asn Thr Gly Pro Asn Trp Ala Thr Phe
705 710 715 720

Ile Lys Arg Phe Pro His Asn Pro Arg Asp Trp Asp Arg Leu Pro Tyr
725 730 735

Leu Asn Leu Pro Tyr Leu Pro Pro Asn Ala Gly Arg Gln Tyr His Leu
740 745 750

Ala Met Ala Ala Ser Glu Phe Lys Glu Thr Pro Glu Leu Glu Ser Ala
755 760 765

Val Arg Ala Met Glu Ala Ala Ala Asn Val Asp Pro Leu Phe Gln Ser
770 775 780

Ala Leu Ser Val Phe Met Trp Leu Glu Glu Asn Gly Ile Val Thr Asp
785 790 795 800

Met Ala Asn Phe Ala Leu Ser Asp Pro Asn Ala His Arg Met Arg Asn
805 810 815

Phe Leu Ala Asn Ala Pro Gln Ala Gly Ser Lys Ser Gln Arg Ala Lys
820 825 830

Tyr Gly Thr Ala Gly Tyr Gly Val Glu Ala Arg Gly Pro Thr Pro Glu

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            835                 840                 845

        Glu Ala Gln Arg Glu Lys Asp Thr Arg Ile Ser Lys Lys Met Glu Thr
            850                 855                 860

        Met Gly Ile Tyr Phe Ala Thr Pro Glu Trp Val Ala Leu Asn Gly His
        865                 870                 875                 880

        Arg Gly Pro Ser Pro Gly Gln Leu Lys Tyr Trp Gln Asn Thr Arg Glu
                        885                 890                 895

        Ile Pro Asp Pro Asn Glu Asp Tyr Leu Asp Tyr Val His Ala Glu Lys
                    900                 905                 910

        Ser Arg Leu Ala Ser Glu Glu Gln Ile Leu Arg Ala Ala Thr Ser Ile
                915                 920                 925

        Tyr Gly Ala Pro Gly Gln Ala Glu Pro Pro Gln Ala Phe Ile Asp Glu
            930                 935                 940

        Val Ala Lys Val Tyr Glu Ile Asn His Gly Arg Gly Pro Asn Gln Glu
        945                 950                 955                 960

        Gln Met Lys Asp Leu Leu Leu Thr Ala Met Glu Met Lys His Arg Asn
                        965                 970                 975

        Pro Arg Arg Ala Leu Pro Lys Pro Lys Pro Lys Pro Asn Ala Pro Thr
                    980                 985                 990

        Gln Arg Pro Pro Gly Arg Leu Gly Arg Trp Ile Arg Thr Val Ser Asp
                995                 1000                1005

        Glu Asp Leu Glu
            1010
```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 3261 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:97..531

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
GGATACGATC GGTCTGACCC CGGGGGAGTC ACCCGGGGAC AGGCCGTCAA GGCCTTGTTC        60

CAGGATGGGA CTCCTCCTTC TACAACGCTA TCATTC GAA GTT AGT TGA GAT CTG         114
                                        Glu Val Ser  *  Asp Leu
                                         1               5

ACA AAC GAT CGC AGC GAT GAC AAA CCT GCA AGA TCA AAC CCA ACA GAT         162
Thr Asn Asp Arg Ser Asp Asp Lys Pro Ala Arg Ser Asn Pro Thr Asp
            10                  15                  20
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2827 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:112..2745

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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GGATACGATG GGTCTGACCC TCTGGGAGTC ACGAATTAAC GTGGCTACTA GGGGCGATAC        60

CCGCCGCTGG CTGCCACGTT AGTGGCTCCT CTTCTTGATG ATTCTGCCAC C ATG AGT         117
                                                         Met Ser
                                                          1

GAC ATT TTC AAC AGT CCA CAG GCG CGA AGC ACG ATC TCA GCA GCG TTC         165
Asp Ile Phe Asn Ser Pro Gln Ala Arg Ser Thr Ile Ser Ala Ala Phe
        5                  10                  15

GGC ATA AAG CCT ACT GCT GGA CAA GAC GTG GAA GAA CTC TTG ATC CCT         213
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Gly Ile Lys Pro Thr Ala Gly Gln Asp Val Glu Glu Leu Leu Ile Pro
20 25 30

AAA GTT TGG GTG CCA CCT GAG GAT CCG CTT GCC AGC CCT AGT CGA CTG 261
Lys Val Trp Val Pro Pro Glu Asp Pro Leu Ala Ser Pro Ser Arg Leu
35 40 45 50

GCA AAG TTC CTC AGA GAG AAC GGC TAC AAA GTT TTG CAG CCG CGG TCT 309
Ala Lys Phe Leu Arg Glu Asn Gly Tyr Lys Val Leu Gln Pro Arg Ser
55 60 65

CTG CCC GAG AAT GAG GAG TAT GAG ACC GAC CAA ATA CTC CCA GAC TTA 357
Leu Pro Glu Asn Glu Glu Tyr Glu Thr Asp Gln Ile Leu Pro Asp Leu
70 75 80

GCA TGG ATG CGA CAG ATA GAA GGG GCT GTT TTA AAA CCC ACT CTA TCT 405
Ala Trp Met Arg Gln Ile Glu Gly Ala Val Leu Lys Pro Thr Leu Ser
85 90 95

CTC CCT ATT GGA GAT CAG GAG TAC TTC CCA AAG TAC TAC CCA ACA CAT 453
Leu Pro Ile Gly Asp Gln Glu Tyr Phe Pro Lys Tyr Tyr Pro Thr His
100 105 110

CGC CCT AGC AAG GAG AAG CCC AAT GCG TAC CCG CCA GAC ATC GCA CTA 501
Arg Pro Ser Lys Glu Lys Pro Asn Ala Tyr Pro Pro Asp Ile Ala Leu
115 120 125 130

CTC AAG CAG ATG ATT TAC CTG TTT CTC CAG GTT CCA GAG GCC AAC GAG 549
Leu Lys Gln Met Ile Tyr Leu Phe Leu Gln Val Pro Glu Ala Asn Glu
135 140 145

GGC CTA AAG GAT GAA GTA ACC CTC TTG ACC CAA AAC ATA AGG GAC AAG 597
Gly Leu Lys Asp Glu Val Thr Leu Leu Thr Gln Asn Ile Arg Asp Lys
150 155 160

GCC TAT GGA AGT GGG ACC TAC ATG GGA CAA GCA ACT CGA CTT GTG GCC 645
Ala Tyr Gly Ser Gly Thr Tyr Met Gly Gln Ala Thr Arg Leu Val Ala
165 170 175

ATG AAG GAG GTC GCC ACT GGA AGA AAC CCA AAC AAG GAT CCT CTA AAG 693
Met Lys Glu Val Ala Thr Gly Arg Asn Pro Asn Lys Asp Pro Leu Lys
180 185 190

CTT GGG TAC ACT TTT GAG AGC ATC GCG CAG CTA CTT GAC ATC ACA CTA 741
Leu Gly Tyr Thr Phe Glu Ser Ile Ala Gln Leu Leu Asp Ile Thr Leu
195 200 205 210

CCG GTA GGC CCA CCC GGT GAG GAT GAC AAG CCC TGG GTG CCA CTC ACA 789

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Pro Val Gly Pro Pro Gly Glu Asp Asp Lys Pro Trp Val Pro Leu Thr
                215                 220                 225

AGA GTG CCG TCA CGG ATG TTG GTG CTG ACG GGA GAC GTA GAT GGC GAC        837
Arg Val Pro Ser Arg Met Leu Val Leu Thr Gly Asp Val Asp Gly Asp
            230                 235                 240

TTT GAG GTT GAA GAT TAC CTT CCC AAA ATC AAC CTC AAG TCA TCA AGT        885
Phe Glu Val Glu Asp Tyr Leu Pro Lys Ile Asn Leu Lys Ser Ser Ser
        245                 250                 255

GGA CTA CCA TAT GTA GGT CGC ACC AAA GGA GAG ACA ATT GGC GAG ATG        933
Gly Leu Pro Tyr Val Gly Arg Thr Lys Gly Glu Thr Ile Gly Glu Met
    260                 265                 270

ATA GCT ATA TCA AAC CAG TTT CTC AGA GAG CTA TCA ACA CTG TTG AAG        981
Ile Ala Ile Ser Asn Gln Phe Leu Arg Glu Leu Ser Thr Leu Leu Lys
275                 280                 285                 290

CAA GGT GCA GGG ACA AAG GGG TCA AAC AAG AAG AAG CTA CTC AGC ATG       1029
Gln Gly Ala Gly Thr Lys Gly Ser Asn Lys Lys Lys Leu Leu Ser Met
                295                 300                 305

TTA AGT GAC TAT TGG TAC TTA TCA TGC GGG CTT TTG TTT CCA AAG GCT       1077
Leu Ser Asp Tyr Trp Tyr Leu Ser Cys Gly Leu Leu Phe Pro Lys Ala
            310                 315                 320

GAA AGG TAC GAC AAA AGT ACA TGG CTC ACC AAG ACC CGG AAC ATA TGG       1125
Glu Arg Tyr Asp Lys Ser Thr Trp Leu Thr Lys Thr Arg Asn Ile Trp
        325                 330                 335

TCA GCT CCA TCC CCA ACA CAC CTC ATG ATC TCC ATG ATC ACC TGG CCC       1173
Ser Ala Pro Ser Pro Thr His Leu Met Ile Ser Met Ile Thr Trp Pro
    340                 345                 350

GTG ATG TCC AAC AGC CCA AAT AAC GTG TTG AAC ATT GAA GGG TGT CCA       1221
Val Met Ser Asn Ser Pro Asn Asn Val Leu Asn Ile Glu Gly Cys Pro
355                 360                 365                 370

TCA CTC TAC AAA TTC AAC CCG TTC AGA GGA GGG TTG AAC AGG ATC GTC       1269
Ser Leu Tyr Lys Phe Asn Pro Phe Arg Gly Gly Leu Asn Arg Ile Val
                375                 380                 385

GAG TGG ATA TTG GCC CCG GAA GAA CCC AAG GCT CTT GTA TAT GCG GAC       1317
Glu Trp Ile Leu Ala Pro Glu Glu Pro Lys Ala Leu Val Tyr Ala Asp
            390                 395                 400

AAC ATA TAC ATT GTC CAC TCA AAC ACG TGG TAC TCA ATT GAC CTA GAG       1365
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Asn Ile Tyr Ile Val His Ser Asn Thr Trp Tyr Ser Ile Asp Leu Glu
405 410 415

AAG GGT GAG GCA AAC TGC ACT CGC CAA CAC ATG CAA GCC GCA ATG TAC 1413
Lys Gly Glu Ala Asn Cys Thr Arg Gln His Met Gln Ala Ala Met Tyr
420 425 430

TAC ATA CTC ACC AGA GGG TGG TCA GAC AAC GGC GAC CCA ATG TTC AAT 1461
Tyr Ile Leu Thr Arg Gly Trp Ser Asp Asn Gly Asp Pro Met Phe Asn
435 440 445 450

CAA ACA TGG GCC ACC TTT GCC ATG AAC ATT GCC CCT GCT CTA GTG GTG 1509
Gln Thr Trp Ala Thr Phe Ala Met Asn Ile Ala Pro Ala Leu Val Val
455 460 465

GAC TCA TCG TGC CTG ATA ATG AAC CTG CAA ATT AAG ACC TAT GGT CAA 1557
Asp Ser Ser Cys Leu Ile Met Asn Leu Gln Ile Lys Thr Tyr Gly Gln
470 475 480

GGC AGC GGG AAT GCA GCC ACG TTC ATC AAC AAC CAC CTC TTG AGC ACG 1605
Gly Ser Gly Asn Ala Ala Thr Phe Ile Asn Asn His Leu Leu Ser Thr
485 490 495

CTA GTG CTT GAC CAG TGG AAC TTG ATG AGA CAG CCC AGA CCA GAC AGC 1653
Leu Val Leu Asp Gln Trp Asn Leu Met Arg Gln Pro Arg Pro Asp Ser
500 505 510

GAG GAG TTC AAA TCA ATT GAG GAC AAG CTA GGT ATC AAC TTT AAG ATT 1701
Glu Glu Phe Lys Ser Ile Glu Asp Lys Leu Gly Ile Asn Phe Lys Ile
515 520 525 530

GAG AGG TCC ATT GAT GAT ATC AGG GGC AAG CTG AGA CAG CTT GTC CTC 1749
Glu Arg Ser Ile Asp Asp Ile Arg Gly Lys Leu Arg Gln Leu Val Leu
535 540 545

CTT GCA CAA CCA GGG TAC CTG AGT GGG GGG GTT GAA CCA GAA CAA TCC 1797
Leu Ala Gln Pro Gly Tyr Leu Ser Gly Gly Val Glu Pro Glu Gln Ser
550 555 560

AGC CCA ACT GTT GAG CTT GAC CTA CTA GGG TGG TCA GCT ACA TAC AGC 1845
Ser Pro Thr Val Glu Leu Asp Leu Leu Gly Trp Ser Ala Thr Tyr Ser
565 570 575

AAA GAT CTC GGG ATC TAT GTG CCG GTG CTT GAC AAG GAA CGC CTA TTT 1893
Lys Asp Leu Gly Ile Tyr Val Pro Val Leu Asp Lys Glu Arg Leu Phe
580 585 590

TGT TCT GCT GCG TAT CCC AAG GGA GTA GAG AAC AAG AGT CTC AAG TCC 1941

Cys Ser Ala Ala Tyr Pro Lys Gly Val Glu Asn Lys Ser Leu Lys Ser
595 600 605 610

AAA GTC GGG ATC GAG CAG GCA TAC AAG GTA GTC AGG TAT GAG GCG TTG 1989
Lys Val Gly Ile Glu Gln Ala Tyr Lys Val Val Arg Tyr Glu Ala Leu
615 620 625

AGG TTG GTA GGT GGT TGG AAC TAC CCA CTC CTG AAC AAA GCC TGC AAG 2037
Arg Leu Val Gly Gly Trp Asn Tyr Pro Leu Leu Asn Lys Ala Cys Lys
630 635 640

AAT AAC GCA GGC GCC GCT CGG CGG CAT CTG GAG GCC AAG GGG TTC CCA 2085
Asn Asn Ala Gly Ala Ala Arg Arg His Leu Glu Ala Lys Gly Phe Pro
645 650 655

CTC GAC GAG TTC CTA GCC GAG TGG TCT GAG CTG TCA GAG TTC GGT GAG 2133
Leu Asp Glu Phe Leu Ala Glu Trp Ser Glu Leu Ser Glu Phe Gly Glu
660 665 670

GCC TTC GAA GGC TTC AAT ATC AAG CTG ACC GTA ACA TCT GAG AGC CTA 2181
Ala Phe Glu Gly Phe Asn Ile Lys Leu Thr Val Thr Ser Glu Ser Leu
675 680 685 690

GCC GAA CTG AAC AAG CCA GTA CCC CCC AAG CCC CCA AAT GTC AAC AGA 2229
Ala Glu Leu Asn Lys Pro Val Pro Pro Lys Pro Pro Asn Val Asn Arg
695 700 705

CCA GTC AAC ACT GGG GGA CTC AAG GCA GTC AGC AAC GCC CTC AAG ACC 2277
Pro Val Asn Thr Gly Gly Leu Lys Ala Val Ser Asn Ala Leu Lys Thr
710 715 720

GGT CGG TAC AGG AAC GAA GCC GGA CTG AGT GGT CTC GTC CTT CTA GCC 2325
Gly Arg Tyr Arg Asn Glu Ala Gly Leu Ser Gly Leu Val Leu Leu Ala
725 730 735

ACA GCA AGA AGC CGT CTG CAA GAT GCA GTT AAG GCC AAG GCA GAA GCC 2373
Thr Ala Arg Ser Arg Leu Gln Asp Ala Val Lys Ala Lys Ala Glu Ala
740 745 750

GAG AAA CTC CAC AAG TCC AAG CCA GAC GAC CCC GAT GCA GAC TGG TTC 2421
Glu Lys Leu His Lys Ser Lys Pro Asp Asp Pro Asp Ala Asp Trp Phe
755 760 765 770

GAA AGA TCA GAA ACT CTG TCA GAC CTT CTG GAG AAA GCC GAC ATC GCC 2469
Glu Arg Ser Glu Thr Leu Ser Asp Leu Leu Glu Lys Ala Asp Ile Ala
775 780 785

AGC AAG GTC GCC CAC TCA GCA CTC GTG GAA ACA AGC GAC GCC CTT GAA 2517

```
Ser Lys Val Ala His Ser Ala Leu Val Glu Thr Ser Asp Ala Leu Glu
            790                     795                     800

GCA GTT CAG TCG ACT TCC GTG TAC ACC CCC AAG TAC CCA GAA GTC AAG      2565
Ala Val Gln Ser Thr Ser Val Tyr Thr Pro Lys Tyr Pro Glu Val Lys
        805                     810                     815

AAC CCA CAG ACC GCC TCC AAC CCC GTT GTT GGG CTC CAC CTG CCC GCC      2613
Asn Pro Gln Thr Ala Ser Asn Pro Val Val Gly Leu His Leu Pro Ala
    820                     825                     830

AAG AGA GCC ACC GGT GTC CAG GCC GCT CTT CTC GGA GCA GGA ACG AGC      2661
Lys Arg Ala Thr Gly Val Gln Ala Ala Leu Leu Gly Ala Gly Thr Ser
835                     840                     845                 850

AGA CCA ATG GGG ATG GAG GCC CCA ACA CGG TCC AAG AAC GCC GTG AAA      2709
Arg Pro Met Gly Met Glu Ala Pro Thr Arg Ser Lys Asn Ala Val Lys
                855                     860                     865

ATG GCC AAA CGG CGG CAA CGC CAA AAG GAG AGC CGC TAACAGCCAT           2755
Met Ala Lys Arg Arg Gln Arg Gln Lys Glu Ser Arg
            870                     875

GATGGGAACC ACTCAAGAAG AGGACACTAA TCCCAGACCC CGTATCCCCG GCCTTCGCCT    2815

GCGGGGGCCC CC                                                        2827
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CLAIMS

1 A birnavirus mutant which is not able to produce a native VP5 protein as a result of a mutation in the VP5 gene of the birnavirus genome.

2 A birnavirus mutant according to claim 1, characterised in that the mutation is a substitution.

3 A birnavirus mutant according to claim 1, characterised in that the mutation is an insertion of a heterologous nucleic acid sequence.

4 A birnavirus mutant according to claim 3, characterised in that the heterologous nucleic acid sequence encodes a polypeptide and the heterologous nucleic acid sequence is under the control of an expression control sequence regulating the expression of the sequence in a cell infected with the virus mutant.

5 A birnavirus mutant according to claims 1-4, characterised in that the birnavirus is infectious bursal disease virus (IBDV).

6 A birnavirus mutant according to claim 5, characterised in that the mutation is in the genome of a virulent field virus.

7 A birnavirus mutant according to claim 5, characterised in that the mutation is in the genome of vaccine strain, preferably in vaccine strain D78.

8 A birnavirus mutant according to claims 5-7, characterised in that the mutant has a mutated start codon and three stop codons in the 5'-end of the VP5 gene as shown in SEQ ID No: 7.

9 A birnavirus according to claims 5-8, characterised in that the IBDV expresses a chimeric VP2 protein comprising virus neutralising epitopes of different antigenic IBDV types.

10 A vaccine against a birnavirus infection in animals, characterised in that it comprises a birnavirus mutant according to claims 1-9 and a pharmaceutically acceptable carrier.

11 A method for determining birnavirus infection in an animal, characterised in that a sample of the animal is examined for the presence of anti-VP5 antibodies.

12 A method according to claim 11, characterised in that the method comprises the steps of:

(i) incubating a sample suspected of containing anti-birnavirus antibodies, with VP5 antigen,

(ii) allowing the formation of antibody-antigen complex , and

(ii) detecting the presence of the antibody-antigen complex.

13 A diagnostic test kit suitable for carrying out a method according to claims 11-12.

14 Use of the lack of the expression of native VP5 protein by a birnavirus mutant as a marker to distinguish vaccinated animals from animals infected with naturally-ocurring birnavirus.

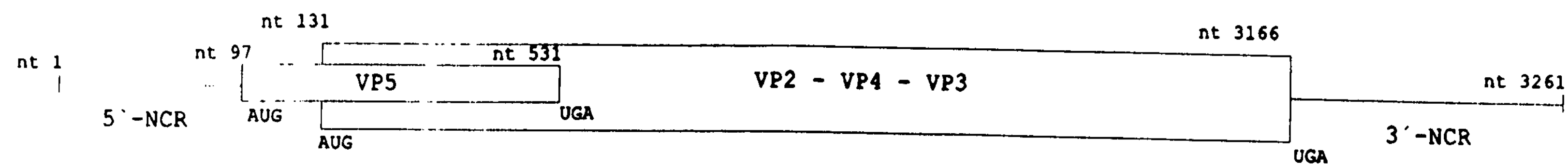
ABSTRACT

The present invention provides a birnavirus mutant which is suited as vaccine candidate in eradication control programmes. The mutant is not able to produce a native VP5 protein, and this feature can be used as a marker to distinguish between animals vaccinated with the VP5 mutant or infected with a naturally-occurring birnavirus.

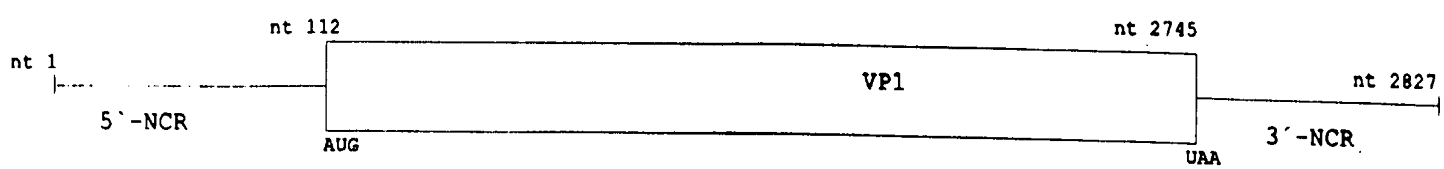
Figure 1

Genomic organization of segment A of strain D78 and segment B of strain P2

D78 segment A



P2 segment B



2/5

<u>Figure 2</u>

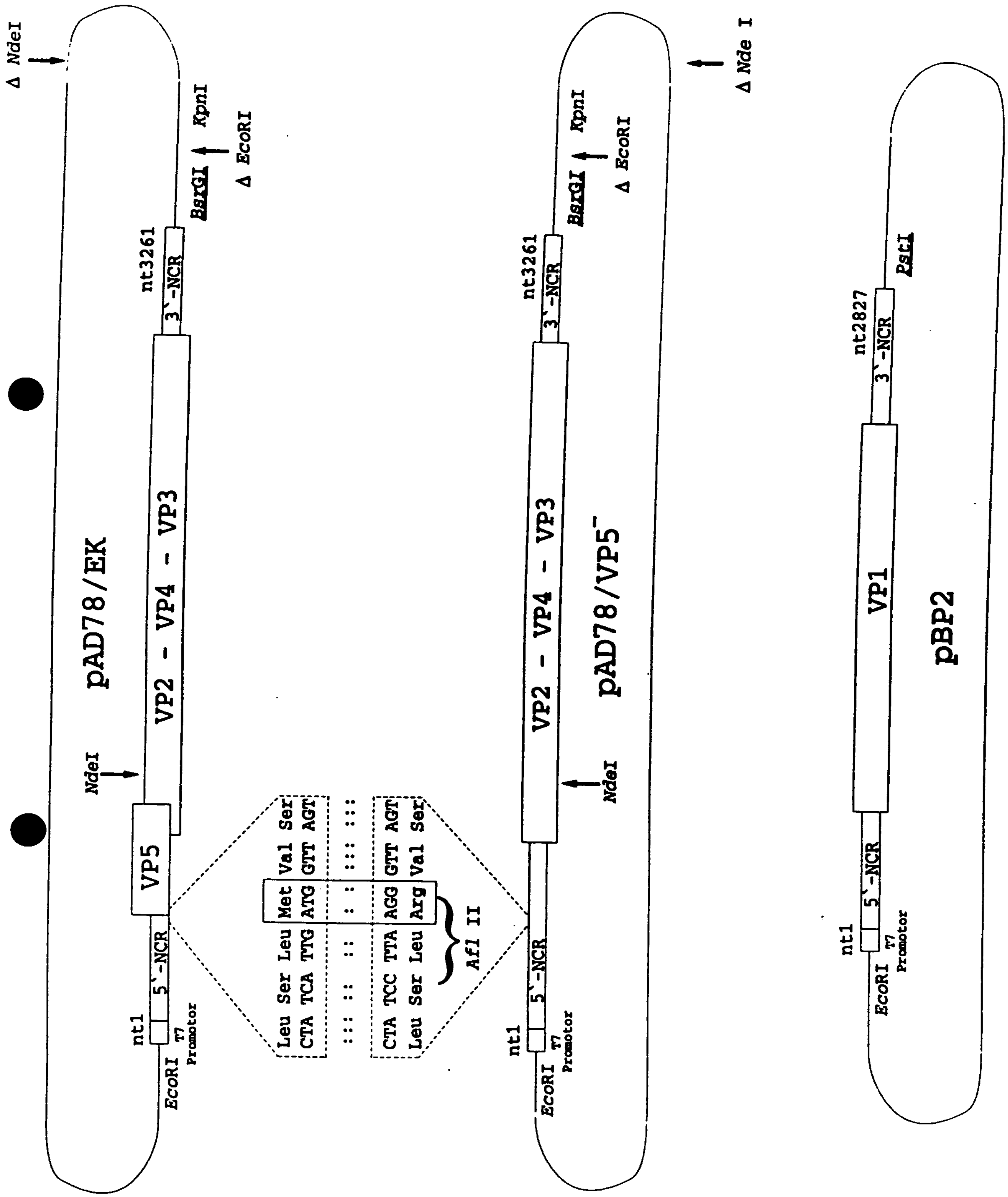

Δ NdeI
KpnI
Δ EcoRI
BsrGI
nt3261
3`-NCR
pAD78/EK
VP2 - VP4 - VP3
NdeI
VP5
5`-NCR
nt1
EcoRI
T7 Promotor
Leu Ser Leu Met Val Ser
CTA TCA TTG ATG GTT AGT
CTA TCC TTA AGG GTT AGT
Leu Ser Leu Arg Val Ser
Afl II
Δ Nde I
KpnI
Δ EcoRI
BsrGI
nt3261
3`-NCR
VP2 - VP4 - VP3
pAD78/VP5⁻
NdeI
5`-NCR
nt1
EcoRI
T7 Promotor
PstI
nt2827
3`-NCR
VP1
pBP2
5`-NCR
nt1
EcoRI
T7 Promotor

Figure 3

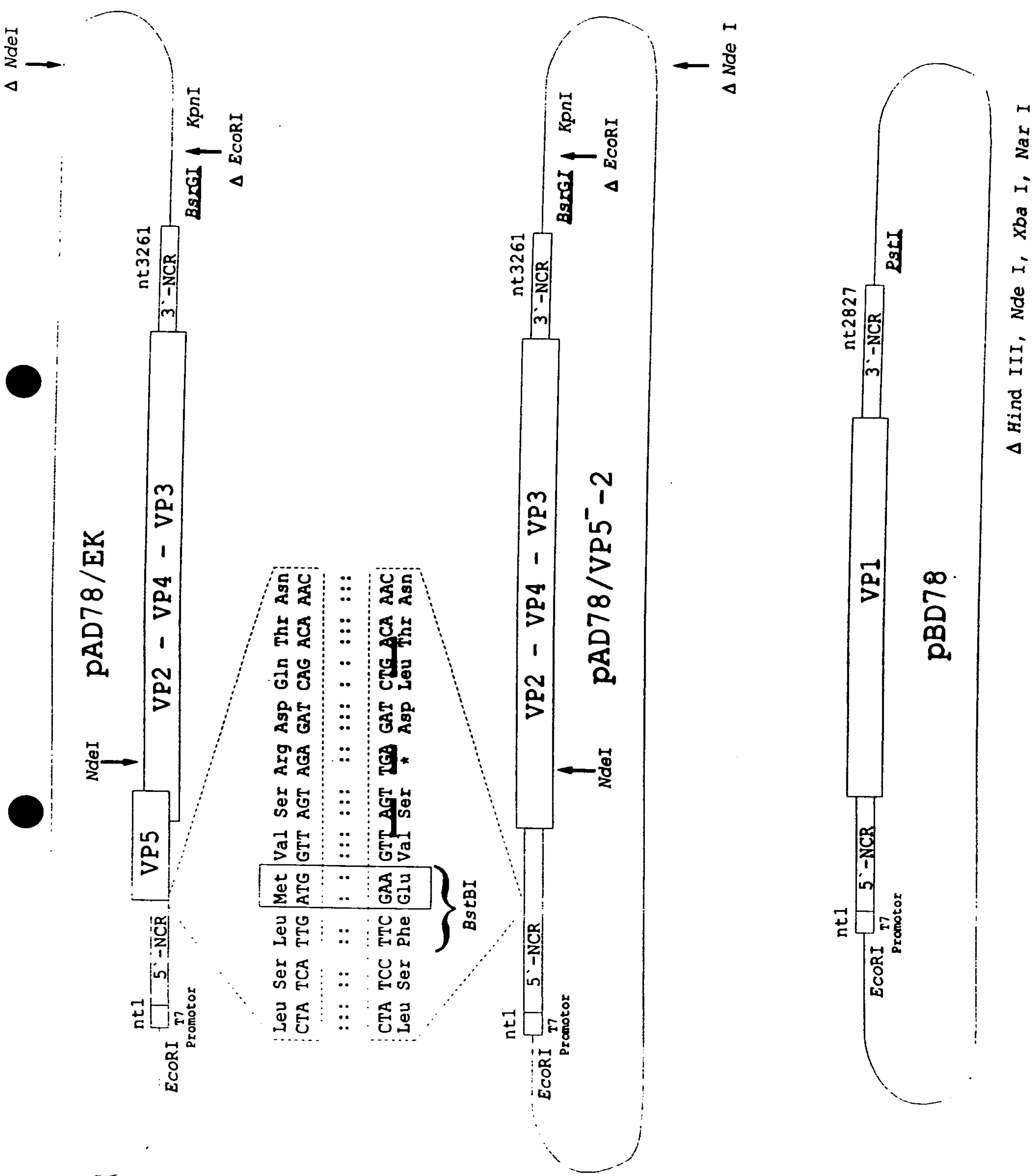

pAD78/EK
Δ NdeI
NdeI
KpnI
Δ EcoRI
BsrGI
nt3261
3`-NCR
VP2 - VP4 - VP3
VP5
5`-NCR
nt1
EcoRI
T7 Promotor
Leu Ser Leu Met Val Ser Arg Asp Gln Thr Asn
CTA TCA TTG ATG GTT AGT AGA GAT CAG ACA AAC
CTA TCC TTC GAA GTT AGT TGA GAT CTG ACA AAC
Leu Ser Phe Glu Val Ser * Asp Leu Thr Asn
BstBI
pAD78/VP5⁻-2
Δ Nde I
pBD78
VP1
nt2827
PstI
Δ Hind III, Nde I, Xba I, Nar I

Figure 4

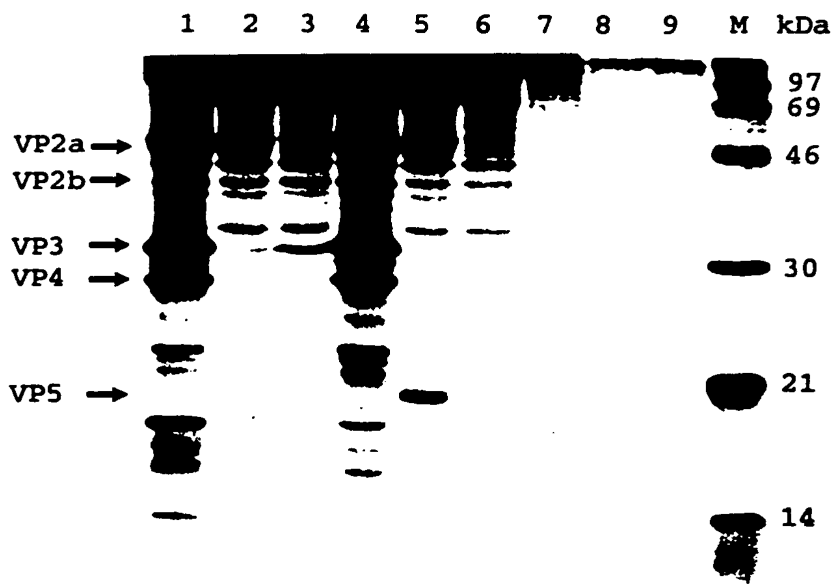
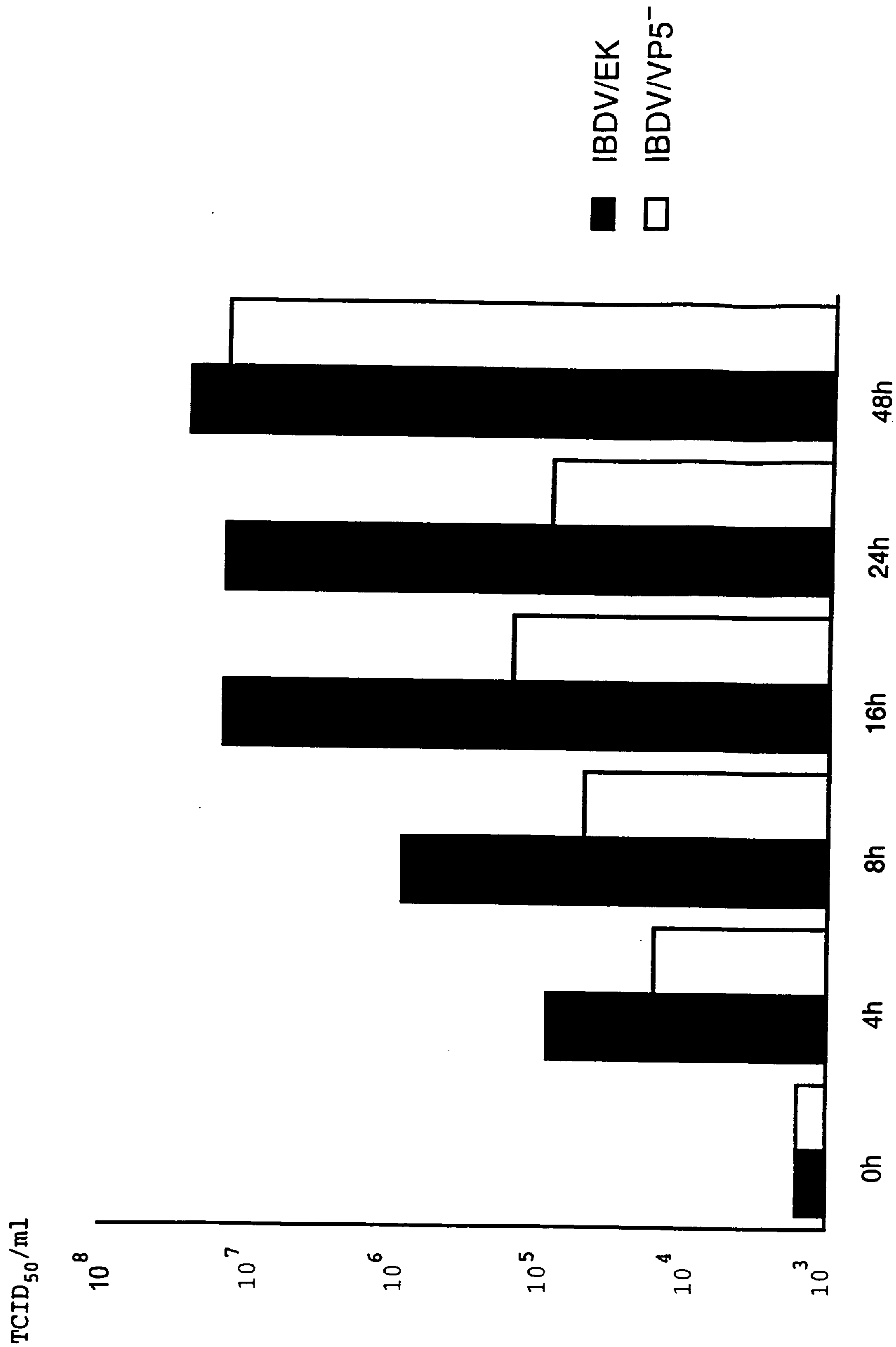


Figure 5


IBDV/EK
IBDV/VP5⁻
$TCID_{50}$/ml
10^8
10^7
10^6
10^5
10^4
10^3
0h
4h
8h
16h
24h
48h